ASSESSING THE EFFECTIVENESS OF WATER QUALITY BEST MANAGEMENT PRACTICES FOR GRAZING-LANDS

A Thesis

by

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ABSTRACT

Best management practices (BMPs) aim to reduce bacterial loading caused by grazing cattle. Relatively little is known about the effectiveness of alternative shade, alternative water, rip-rap, and prescribed grazing as potential BMPs. Prescribed grazing evaluated how stocking rate affected bacterial loading. *E. coli* concentrations in runoff samples were compared between plots with various stocking rates. GPS collars were used to determine how a shade pavilion, water source, or rip-rap effected cattle distribution within a stream and riparian pasture by comparing time cattle spent at a location before and after implementing the BMPs. While plots were stocked or within 14 days of being destocked, *E. coli* concentrations were significantly higher than destocked pastures. No significant differences were observed between *E. coli* concentrations in runoff from heavily stocked, moderately stocked, or non-grazed pastures when pastures had been destocked for greater than 14 days. On average, the shade structure reduced cattle's dependence on riparian shade by 30%. The alternative water BMP did not reduce the amount of time cattle spent within the riparian zone; however, the study was limited to one trial. Riparian rip-rap trials were inconclusive; however, preliminary rip-rap trials showed 20 to 40 cm diameter rip-rap was effective at modifying cattle trough preference.

Advances in microbial source tracking, specifically *Bacteroides*, have allowed better identification of bacterial sources. However, genetic variability within some *Bacteroides* sequences may undermine the accuracy of these molecular markers. Localized gene-copy curves were created from 12 bovine fecal samples from a single

herd, and qPCR assays were used to determine if they better correlated *Bacteroides* and *E. coli* populations. Sequences were pyro-sequenced to see if mismatches occurred within primer/probe regions. Base-pair mismatches occurred, and affected qPCR efficiencies. Fecal pollution load estimations were overestimated by using sequences with more mismatches. Genetic diversity was observed within samples from all locations, and indicated genetic variability within *Bacteroides* populations occurs within a single location as much as between locations. Thus, creating standard curves for individual watersheds would not necessarily improve pollution load estimations.

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CHAPTER I

INTRODUCTION

Riparian health and stream water quality are intricately linked and important to the sustainability of in-stream contact recreation, aquatic life habitat, and fishing. Water pollution has been a prominent environmental concern since the late 1960s. According to the Environmental Protection Agency (EPA, 2011), there are 16,608 km (10,320 mi) of impaired rivers and streams known in Texas. Over half of these streams are impaired from non-point sources (NPSs) such as urban runoff, avian and non-avian wildlife, grazing, irrigated cropland, mining, and others. Agricultural operations have been cited for contributing over 20% of all the in-stream pollutants in Texas (EPA, 2011). To help mitigate pollution, state and federal agencies have initiated total maximum daily loads (TMDLs) or watershed protection plans (WPPs) to target needed water quality best management practices (BMPs) to reduce pollutant loading.

To successfully address water quality impairments, effective BMPs and source tracking methods are two crucial areas needing further development. Developing BMPs to reduce or prevent water pollution is essential. Much time, money, energy, and frustration might be saved by knowing the effectiveness and limitations of a particular BMP before investing and implementing BMPs. Source tracking is the practice of determining a pollutant's origin. Without first determining the pollutant source and contribution percentages, implementing water-quality improvement BMPs may be a futile task. Source tracking not only pinpoints which pollutant sources need to be

controlled, but it also allows researchers to determine specific BMP successes or failures between source groups.

CHAPTER II

GRAZING-LAND BMPs

2.1 Contaminant Fate Modification

Much work has been done in examining the effects of livestock on riparian health and water quality (Clary and Kinney, 2002; Giuliano, 2006; Line, 2003; Sovell et al., 2000). Other studies have examined the link between the proximity of contaminant deposition and in-stream water quality. It is generally recognized the shorter the distance between contaminant deposition and the waterway, the greater the negative effect on water quality (Larsen et al., 1994). In an attempt to control contaminant deposition and fate processes, structural BMPs have been implemented to modify animal behavior. Specifically, cattle travel and grazing patterns have been modified resulting in altered fecal deposition patterns. In the past, researchers were limited to visual observation to collect the spatial position of grazing livestock or fecal deposits (Miner et al., 1992; Sheffield et al., 1997). With Global Positioning Systems (GPS) technology, not only can more data be collected, but it is often more accurate, and allows cattle location to be observed in the context of a herd and at all hours of the day. GPS data points taken at evenly spaced time-intervals can be used to correlate the amount of time cattle spend within a given area (Koostra et al., 2003), and fecal deposition is acknowledged to be directly correlated to the time cattle spend at any given location (Lange and Willcocks, 1978).

Some common BMPs used to reduce pollution from livestock grazing operations include riparian buffer strips, exclusion fencing, prescribed grazing, off-stream water

sources, and rotational stocking. Despite the variety of BMPs available, there is still need for development and testing of additional, cost-effective BMPs. This is because the landscape and operations BMPs are intended to facilitate are highly diverse. Producers need BMPs relevant to their operation that will not negatively impact production. For this reason, there should be an assortment of BMPs producers could select and implement appropriate for their specific situations.

One BMP that has met much resistance from cattle producers is exclusion fencing (Dulay, 2012). Exclusion fencing is the practice of fencing off the stream and riparian zone to prevent livestock from grazing and watering within the riparian zone and waterway. While it has proven very effective at keeping livestock out of the riparian zone, and has been shown to reduce bacterial and nutrient loading in some cases (Line et al., 2000; Line, 2003), its use has been highly unpopular among stakeholders. From a ranch management perspective, it is costly (Clawson, 1993), labor intensive, overly restrictive (McIver, 2004), and not always effective (Homyack and Giuliano, 2002). While many stakeholders agree environmental stewardship is very important, opposition exists because the BMP offers little practical benefit from a ranch productivity or management standpoint (Bewsell and Kaine, 2005; Hejna et al., 2007).

Water quality BMPs providing more practical and diversified benefits from a farm/ranch management context encourage higher adoption rates (Kim et al., 2004). Since BMPs are primarily voluntary, stakeholder acceptance is critical. It is necessary to provide stakeholders with simple, cost-effective BMPs beneficial to the agricultural operation (Bewsell and Kaine, 2005). For this reason, prescribed grazing, alternative

shade, alternative water, and rip-rap are BMPs having been suggested as alternatives to exclusion fencing (Wagner et al., 2008). These BMPs are thought to offer similar water quality benefits without the drawbacks of exclusion fencing, and they include additional ranch-related benefits such as soil conservation (Clinton and Vose, 2003) and improved pasture utilization (Wagner et al., 2010). Still, relatively little is known about the effectiveness of these BMPs with the existing literature either being limited, inconclusive, or inconsistent (Bryant, 1982; Sheffield et al., 1997).

2.1.1 Alternative Water

While some literature exists regarding the effectiveness of alternative water sources for livestock as a water quality BMP, results are often conflicting. One study (Bryant, 1982) did not observe any appreciable difference in the amount of time cattle spend within the riparian zone when using an alternative water source. However, two other studies have shown an alternative water source reduced the amount of time cattle spend watering within the stream by around 90% (Miner et al., 1992; Sheffield et al., 1997). Furthermore, after implementing an alternative water source, one study found riparian erosion was reduced by 77% (Sheffield et al., 1997).

2.1.2 Alternative Shade

In pastureland, much of the natural shade is often located along the riparian zone. In the summer months, cattle seek shade to cool off (West, 2003). Temperature and relative humidity have been found to be two of the main driving factors for cattle's response to seeking shade (Bryant, 1982). Byers (2004) observed cattle spent 80% of

their time in the shade while in the riparian zone. Providing an alternative shade source outside of the riparian zone has been suggested as a potential water quality BMP for grazing-lands (Agouridis et al., 2004; Andrae et al., 2005; Byers, 2004). However, few studies have evaluated the effectiveness of alternative shade at modifying cattle behavior; thus, this remains a BMP that should be studied to a greater extent (Agouridis et al., 2005). Most shade studies have been primarily focused on optimizing metabolism or milk production in cattle (Blackshaw and Blackshaw, 1994) rather than providing water quality benefits.

One Geographical Information Systems (GIS) study testing the effectiveness of an alternative shade structure concluded it "did not decrease the amount of time cattle spent along the streambanks" (Agouridis et al., 2004). However, Agouridis et al. (2004) conceded the lack of treatment effects may have been due to data constraints. Another possible reason for this may be due to the shade configuration at the study site. The presence of non-riparian shade trees (Koostra et al., 2003) may confound the results because the trees act as a natural BMP. For this reason, the control data from this study may not have varied significantly from the treatments. This may explain why the alternate shade BMP results of the study (Agouridis et al., 2004) were ineffective at reducing the amount of time cattle spent in or near the stream. This underscores the importance of proper placement of alternative shade structures as abundant natural non-riparian shade may negate the necessity and compromise the effectiveness of an alternative shade structure.

2.1.3 Rip-Rap

Rip-rap, or irregularly shaped rocks, is commonly used as a BMP for streambank stabilization and erosion control (Seaberg et al., 1990); however, its use in livestock behavior modification has not been documented. The premise behind using rip-rap to modify cattle grazing and stream-crossing behavior is the rip-rap will act much like a cattle guard. It is believed cattle will choose not to cross the hard, uneven, and unsecure rip-rap because they prefer stable footing and level ground. By strategically placing rip-rap at key stream-crossing sites, cattle travel behavior may be altered to help stabilize streambanks, minimize erosion, or even reduce pollution loading by reducing the amount of time cattle spend within the riparian zone. There is no evidence in existing literature indicating the effectiveness of using rip-rap in this regard; however, it has been suggested rip-rap may serve as an effective cattle deterrent and water-quality BMP (Redmon et al., 2011). This is supported by one study that found cattle tended to avoid grazing in areas with more than 30% rock cover (Hohlt et al., 2009). Other studies have observed additional benefits of rock cover such as increased water infiltration rates (Dadkhah and Gifford, 1980; McCalla et al., 1984). Rip-rap may also serve as a potential alternative to fenced water gaps in some cases. It is presumed rip-rap size, shape, percent ground cover, and distribution width will affect BMP effectiveness.

2.1.4 Prescribed Grazing

Overstocked pastures are typically more susceptible to invasion of non-native grasses, invasive shrub species (Silvertown et al., 1994), increased bacteria and nutrient loading, and erosion (Hubbard et al., 2004). Conversely, proper management of grazing

lands promotes healthier pastures (Campbell, 1966; Hubbard et al., 2004). Prescribed grazing practices have been associated with water quality benefits such as reduced bacteria, nutrients (Simon and Collison, 2002), and erosion (Lyons et al., 2000). Prescribed grazing attempts "to manage grazing animals to maintain adequate vegetation cover on sensitive areas" such as riparian zones (NRCS, 2006). Prescribed grazing also provides water quality benefits by maintaining well-established grasslands acting as grass buffer strips and may encourage water infiltration.

Time, duration, and intensity of grazing events are factors affecting bacteria and nutrient loading in runoff water. Grazing intensities and stocking rates differ among cattle operations due to differences in forage type and regional precipitation rates (Oesterheld et al., 1998). Wagner et al. (2010) observed higher *Escherichia coli* counts in runoff when stocking rates were heavier than 4 ha/AU (10 ac/AU). Wagner et al. (2010) further observed *E. coli* counts in runoff from lighter stocked pastures (i.e. below 4 ha/AU (10 ac/AU)) did not vary significantly from those of non-grazed pastures. Supporting the conclusions of other studies, Wagner et al. (2010) reported a significant decrease in bacterial loadings in runoff samples within two weeks of removing cattle from the pasture (Gary et al., 1983; Sovell et al., 2000). Nevertheless, more work is required to understand the correlation between bacterial loading and grazing events, stocking rates, and runoff events for the prescribed grazing BMP.

2.2 Objectives and Hypotheses

The over-arching goal of this study is to help determine the applicability and effectiveness of certain structural and non-structural water-quality BMPs in reducing bacterial loading to grazing-land streams. Specific objectives included the following:

2.2.1 Non-structural BMPs

- Determine if prescribed grazing reduces bacterial loading and concentrations
- Determine if correlations exist between bacterial loading and concentrations and time between runoff events and grazing events

H_0: Bacterial concentrations in runoff will not vary significantly between grazing plots

H_1: Bacterial concentrations in runoff from the heavy grazed plots will be higher than those of the prescribed and non-grazed plots, and the prescribed grazed plots will be greater than the non-grazed plots

2.2.2 Structural BMPs

- Determine if alternative water, alternative shade, and rip-rap affect cattle behavior and reduce the amount of time they spend within the waterway and riparian zone

H_0: Implementation of alternative water, alternative shade, and rip-rap will not significantly change the movement and distribution of cattle.

H_1: Implementation of alternative water, alternative shade, and rip-rap will reduce the amount of time the cattle spend at or near the stream.

2.3 Methods

2.3.1 Prescribed Grazing- Site Descriptions

Prescribed grazing was evaluated at locations near College Station, Sinton, and Riesel, TX (Table 2.1 and Fig. 2.1). The test site near College Station is located on the Texas A&M University Beef Cattle Systems Center (BCSC) west of the Brazos River (30°31’47”N, 96°24′53″W). Another test site is located near Sinton, TX on the Rob and Bessie Welder Wildlife Refuge (WWR) west of the Aransas River (28° 6'56"N, 97°21'21"W). Each test site had three one-hectare plots with edge-of-field automated samplers. Berms and fences had been installed around the outer-perimeter of each plot to prevent grazing and runoff from outside the plot from contaminating storm-water samples for previous studies. Plots were managed with three stocking rates consisting of heavy stocking, appropriate stocking as determined by prescribed grazing strategies, and non-grazing. Runoff samples were also collected from two sub-watersheds at the USDA Agriculture Research Service (ARS) in Riesel, Texas (SW12 and W10) and enumerated for *E. coli* concentrations.

2.3.2 Grazing Strategies

Stocking rates and grazing events were determined on the basis of available forage. Ideally, the three prescribed grazing plots were grazed according to the regionally appropriate moderate stocking rate; however, environmental factors limited

forage availability, and grazing events and stocking rates were reduced in duration or intensity. The stocking rate at the heavy-stocked plots was double that of the prescribed grazing, and grazing events for the heavy-stocked plots coincided at the same time as the prescribed grazed plots. The non-grazed plot served as the control to determine background bacterial concentrations. Stocking rate and duration were recorded for each grazing event.

Table 2.1 Locations and characteristics of watershed sites. Wagner (2011)

Site	Lat/Long	Vegetation	Management	Soil Types	Hydrologic Soil Group	Slope
Beef Cattle Systems Center, College Station, Texas						
BB1	30° 31'44.3"N /96°24'58.3"W	Tifton 85 bermudagrass	Ungrazed	Belk clay	D	0.20%
BB2	30° 31'47.5"N /96°24'57.7"W	Tifton 85 bermudagrass	Properly Stocked	Belk clay	D	0.20%
BB3	30° 31'47.7"N /96°24'57.9"W	Tifton 85 bermudagrass	Overstocked	Belk clay	D	0.20%
Welder Wildlife Foundation, Sinton, Texas						
WWR1	28° 6'55.97"N /97°21'20.82"W	Native rangeland	Ungrazed	Victoria & Monteola clay	D	3.30%
WWR3	28° 6'52.60"N / 97°21'13.83"W	Native rangeland	Properly stocked	Victoria & Monteola clay	D	0.80%
USDA-Agricultural Research Service (ARS) Riesel Watersheds, Riesel, Texas						
SW12	31° 28'48"N / 96° 52'59"W	Native prairie	Ungrazed	Heiden & Houston Black clay	D	3.80%
W10	31° 27' 12"N / 96° 52' 48" W	Bermudagrass	Properly stocked	Heiden & Houston Black clay	D	2.6%

2.3.3 Runoff Sample Collection

ISCO automated samplers, bubble flow meters, and V-notch weirs were used to collect composite edge-of-field runoff samples. During runoff events, automated-samplers activated and collected 50 ml (1.7 oz) of storm-water runoff for each 4.25 m^3

(150 cf) flowing through the weir. The sampler was activated when the water level exceeded 6 mm (0.02ft). For each plot, the composite water sample was collected in a 15 L polyethylene (3.96 gal) bottle.

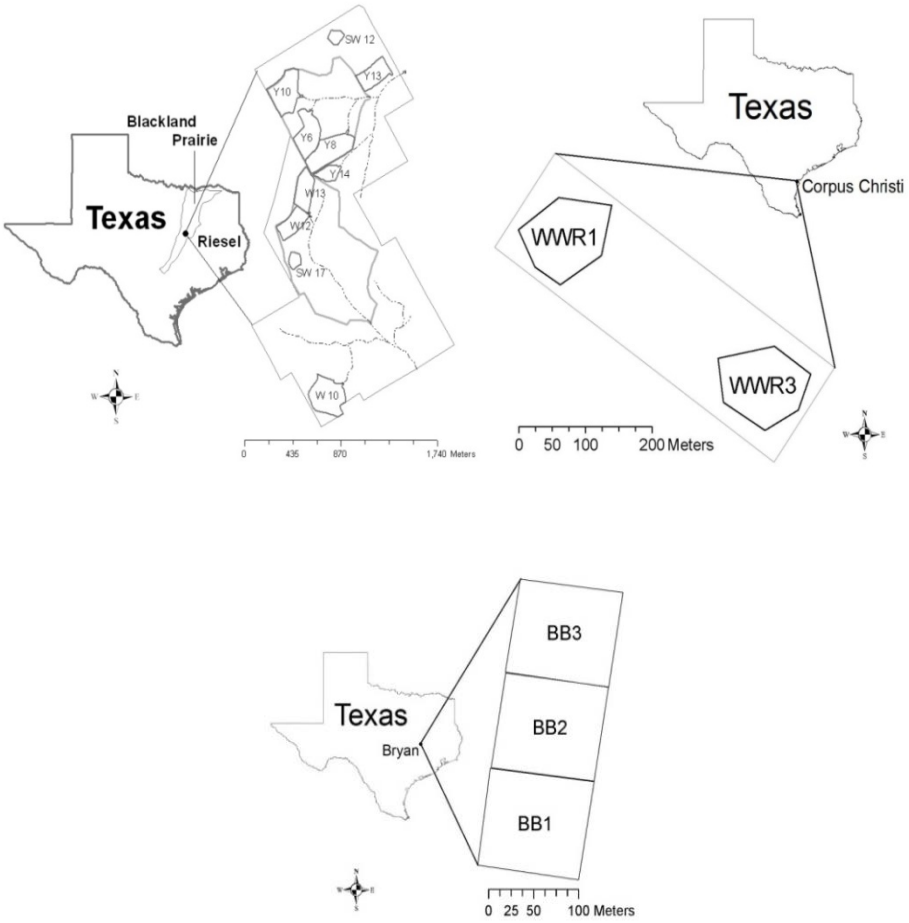

Figure 2.1. Riesel watersheds (top left), Welder Wildlife Refuge watersheds (top right), and Beef Cattle Systems Center 1-ha watersheds (bottom center)

Bottles were washed with deionized water and surface disinfected using ultra violet light prior to placement in the Isco automated samplers. A stilling well and bubble flow meter were used in conjunction with the v-notch weir to determine the total amount of flow for a given runoff event. The total volume of each runoff even was recorded to obtain the flow-weighted bacterial load and event mean concentration of each runoff event. Water samples were collected and processed within six hours of the end of the sampling event. Each sample was transferred to a sterile 500 ml (16.9 oz) bottle and preserved on ice until analysis to prevent bacterial die-off.

2.3.4 Enumerating Bacteria

Water samples were enumerated for *E. coli*, *Enterococcus*, and fecal coliform bacteria using EPA Methods 1603 (EPA, 2003) and 1600 (EPA, 2006) and Standard Method 9222D (WPCF, 1989) respectively. Bacterial counts were recorded for all three bacteria types, and multiplied by the total volume of runoff to obtain the total bacterial load. Runoff samples were compared between locations and sites, against stocking rate in hectares per animal unit year (ha/AUY), and against the number of days since grazing to determine if correlations exist between any of these factors.

2.3.5 Structural BMPs- Site Description

The alternative shade, alternative water, and rip-rap BMPs were evaluated at the Texas AgriLife Research Center at McGregor, Texas (Fig 2.2). The study site is a 28.7 ha (71 ac) grazed pasture with an intermittent headwater stream of the South Bosque River flowing through it. An estimated 6% of the pasture area was vegetated with trees

large enough for shade coverage. Shade was almost exclusively limited to within the riparian zone. The pasture was provided with an off-stream water trough at the southeast corner of the pasture. The pasture had been heavily stocked, and there was evidence of stream-bank erosion at sites where cattle frequently crossed the creek. Six to eight Lotek ® GPS 3300LR collars (Lotek Wireless Inc., Newmarket, Ontario, Canada) were placed on randomly-selected cows and used to record their locations over five 21 to 23 day trials. Each GPS collar was calibrated to take a single locational data-point every five minutes. The creek, pasture boundaries, and the riparian zone were delineated using remote sensing.

2.3.6 Alternative Water and Shade

Before beginning trials, GPS collars were placed on cattle, and the cattle were released into the study pasture. The collars were programmed to begin collecting GPS data-points on the midnight after the cattle were turned into the pasture. Data points were collected at each five minute interval for the remainder of the trial. The first 10 to 12 days of each 21 to 23 day trial served as the control period in which the GPS collars were initiated to monitor cattle location prior to BMP implementation. Halfway through the trial, the BMP was implemented (i.e. shade cloth was erected and/or water trough provided), and the collars continued to collect data-points for another 10 to 12 days. This 'post implementation' period served as the treatment period; allowing cattle behavior to be compared between BMP treatment and control periods. The alternative water source was located 145 m (476 ft) from the creek at its closest point. The alternative water trial was performed while water was flowing in the creek. A 9.1 x 9.1

m (30 x 30 ft) shade pavilion with shade cloth was erected for the alternative shade BMP. The shade structure was placed approximately 140 m (459 ft) away from the creek and riparian zone where other large trees could serve as potential shade locations for cattle. Three trials were conducted for the spring, summer, and fall months to help determine how seasonal variances affect the cattle's behavior to seek shade. To minimize confounding results, the alternative shade trials were conducted at different times than the alternative water trial.

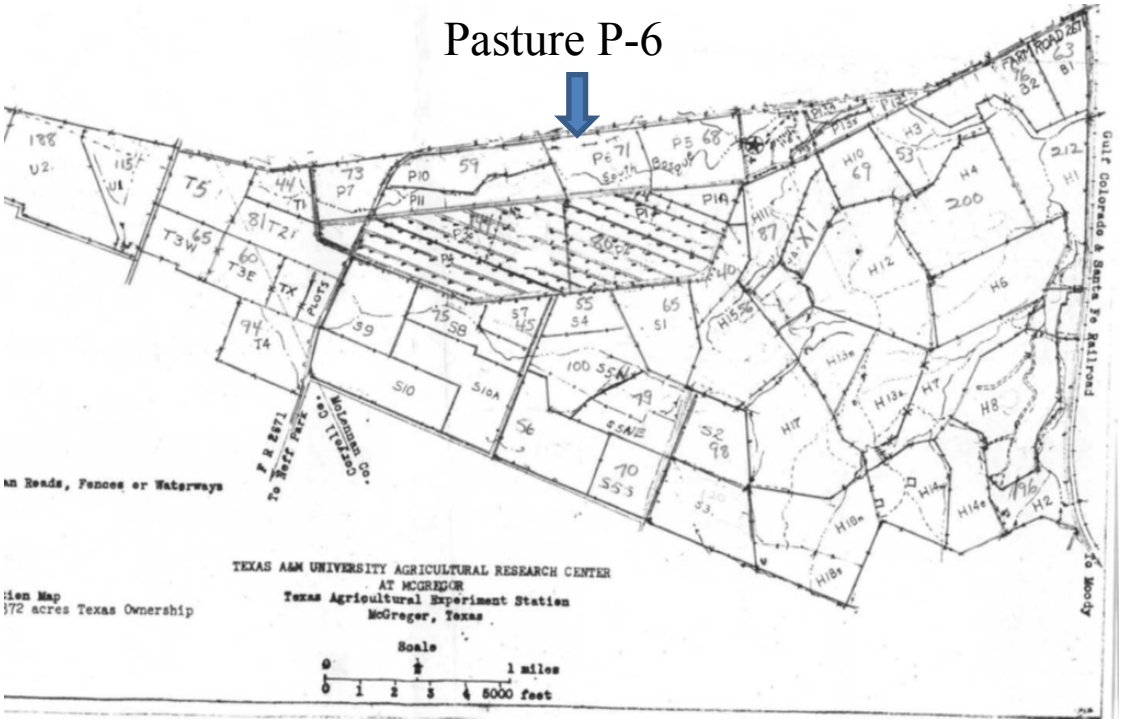

Figure 2.2. McGregor BMP study pasture, P-6. (TSSWCB, 2012)

2.3.7 Rip-rap

Rip-rap was evaluated using: (1) preliminary trials consisting of four day-long visual observations and (2) a riparian rip-rap trial consisting of three GPS trials. The preliminary trials served to determine the needed size and dimensions of an effective rip-rap treatment. The most effective rip-rap treatment, as determined by the preliminary trials, was then implemented in a riparian setting at the McGregor study pasture and analyzed with the GPS collars. These trials were conducted in an attempt to quantify changes in cattle behavior due to installation of rip-rap in a riparian setting.

Preliminary trial observations began before daybreak and lasted until after sunset. Two water troughs were set up approximately 25 m (82 ft) apart from each other in a pasture adjacent to the BCSC test plots. Troughs were located away from fences to allow cattle access from all directions. One trough ($T_{rip\text{-}rap}$) was treated with rip-rap while the other trough ($T_{control}$) remained untreated and served as the control. Other water troughs in the pasture were drained prior to observations making $T_{rip\text{-}rap}$ and $T_{control}$ the only two water sources available to the cattle. Rip-rap was implemented surrounding $T_{rip\text{-}rap}$ at a distance of 2 m (6 ft) from the perimeter of the trough. Rip-rap from 10 to 20 cm (4 to 8 in) in diameter was the first size implemented. The following three trials assessed 20 to 40 cm (8 to 16 in) diameter rip-rap. Percent ground cover was estimated to exceed 85%. To provide an acclimation period, cattle were released into the pasture on the day prior to the observation. Observations were taken from a parked vehicle approximately 40 m (130 ft) from either trough.

For the riparian rip-rap trial, frequently used cattle stream crossings, identified utilizing results of prior GPS trials, were lined with 20 to 40 cm (8 to 16 in) diameter rip-rap in an attempt to deter cattle away from using these crossings along the stream and riparian zone. Rip-rap was not available in the size desired, so two sizes were used. The first load ranged from 30 to 76 cm (1 to 2.5 ft) in diameter while the second load ranged from 10 to 20 cm (4 to 8 in) in diameter. Over 50 cubic meters (approximately 55 tons) of rip-rap was used to line and enclose a 40 m (130 ft) section of stream. Ideal spreading of rip-rap was impeded by large trees and unevenly sloped streambanks. In general, rip-rap was piled between 30 to 60 cm (1 to 2 ft) high and between 90 to 150 cm (3 to 5 ft) wide. Rip-rap was implemented with a front-end loader and, in some cases, spread by hand.

2.3.8 Statistical Analysis

For the non-structural BMP, *E. coli* concentrations were compared by site, location, stocking rate (ha/AUY), and days since grazing. All comparisons were analyzed either using SAS or Sigma Plot statistical software. Dunn's method was used to compare multiple pair-wise differences between sites and locations. The level of significance was set at an alpha value of 0.05. Comparisons between sites and locations were analyzed using an analysis of variance. Treatment effects between locations associated with stocking rates and time between grazing events and runoff events were grouped together into categories and compared using an analysis of variance. All bacteria data was log transformed prior to analysis. Median values with a statistical significance smaller than 5% ($P<0.05$) were determined to be significantly different.

The alternative shade and alternative water structural BMPs were analyzed by counting the number of data-points within the different buffer zones (i.e. riparian zone, water trough, and shade pavilion) before and after BMP implementation. At the end of each trial, GPS collars were taken off and data downloaded. The GPS data was mapped in ArcMAP and used to count the number of points within each buffer zone. Data-points were first normalized to account for differences in the total number of data-points collected before or after BMP implementation (see equations 2.1 and 2.2). The percent differences between 'Pre' and 'Post' BMP periods were then calculated using equation 2.3.

$$\frac{No.of\ points\ prior\ to\ BMP\ at\ buffer\ zone}{Total\ points\ prior\ to\ BMP} = \%\ Pre \qquad (2.1)$$

$$\frac{No.of\ points\ post\ BMP\ at\ buffer\ zone}{Total\ points\ post\ BMP} = \%\ Post \qquad (2.2)$$

$$\frac{\%\ Post - \%\ Pre}{(\%\ Pre)} * 100 = \%\ Diff \qquad (2.3)$$

For the rip-rap BMP, the previously obtained data points from the alternative shade and water GPS trials served as the pretreatment period and provided control data for the riparian rip-rap trials. Using ArcMAP, a 10 m (32.8 ft) buffer was created extending 5 m (16.4 ft) either direction from the center of the stream. The 10 m stream buffer was then separated into 10 m block segments as seen in Fig. 2.3. Data-points within the 10 m blocks were counted for each trial and used to represent frequently used

stream crossings. The number of data points observed within the blocks were then compared between trials to determine how rip-rap effected cattle behavior along the riparian zone.

Figure 2.3 McGregor 10 m by 10 m segment stream buffer blocks

2.4 Results and Discussion

Fifty-eight runoff samples were collected and enumerated for *E. coli* concentrations from 9 sites between the Riesel, BCSC, and Welder locations from June of 2010 to June of 2012. A total of 44 fecal coliform and *Enterococcus* concentrations were collected and enumerated from 6 sites at the BCSC and Welder locations. To broaden the duration and scope of the overall prescribed grazing study, the *E. coli* concentration data was combined with the 127 *E. coli* concentrations results of the Wagner 2011 study (Appendix B).

2.4.1 Time and Stocking Rate Correlations

Regression analysis of *E. coli* concentrations and stocking rate and time between grazing events and runoff events yielded poor results; however, grouping the *E. coli* results into the three main treatment categories and comparing with an analysis of variance yielded much more conclusive results. Higher *E. coli* concentrations were observed when runoff events occurred while cattle were stocked within the field plots, or at least within 14 days of being destocked (Fig. 2.4). Compared against stocked field plots, average *E. coli* concentrations were reduced by 57% when field plots were destocked for greater than 14 days, while non-grazed plots showed a 69% reduction from actively stocked plots. The best evidence there is a significant effect between the days since grazing events and *E. coli* concentrations can be seen in Fig. 2.4 There is a significant difference between *E. coli* concentrations when sites are stocked verses when they are destocked or non-grazed ($P = <0.001$). Differences between destocked sites and non-grazed sites were insignificant. This was expected as a large percentage of *E. coli*

are generally considered to die-off after the first 14 days outside of the host (Gary et al., 1983; Sovell et al., 2000).

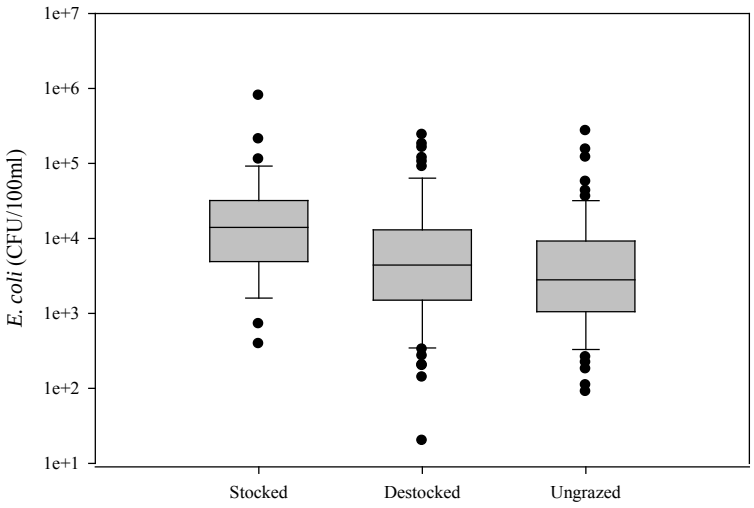

Figure 2.4 *E. coli* concentrations compared between stocked, destocked, and non-grazed runoff events. Stocked represents all runoff events occurring during a grazing event or at least within 14 days of being destocked. Destocked represents all runoff events occurring after 14 days of being destocked. Non-grazed represents runoff events occurring on controlled sites that received no grazing treatment. Lines within the box represent the median value. The upper and lower bounds of the box represent the 75th and 25th percentile respectively. Similarly, the upper and lower whiskers represent the 90th and 10th percentile respectively. Outliers are represented by filled circles.

It is interesting the destocked and, particularly, the non-grazed sites have such a large range of *E. coli* concentrations. This is troubling because although wildlife contributions were considered to play some role in *E. coli* loading, it was not expected background concentrations would be so large. Wildlife were occasionally observed within or near the field plots, it is possible the wildlife presence may have caused the large *E. coli* concentrations seen in this study. Wildlife have been identified as a

significant source of *E. coli* in some agricultural watersheds (Somarelli et al., 2007).

On several occasions, large flocks of Meadowlarks were observed within the field plots at the BCSC sites. It is likely other avian wildlife were also present within the field plots over the duration of the study and could have contributed to microbial concentrations in runoff samples. Other possible fecal contributors to the BCSC field plots include coyotes, mice and other small rodents, feral hogs, and even domestic canines. Coyotes and feral hogs have been seen and/or heard on several occasions within a mile of the field plots. Large canine footprints were observed immediately adjacent to the field plots following multiple rainfall events. At the Welder Wildlife Refuge, the number of possible wildlife contributors is much broader. Deer and hog footprints and trails were regularly observed within the Welder field plots. Similarly, wildlife are the most probable cause for the high bacterial concentrations at the Riesel non-grazed and destocked sites.

It is possible *E. coli* can persist or even replicate within the soil (Gagliardi and Karns, 2000). Moreover, introduced *E. coli* have been observed to aggregate within the dispersible clay fraction (Recorbet et al., 1995). All three experimental locations have high clay-content soils. If the high *E. coli* concentrations observed at non-grazed and destocked sites are not directly due to wildlife contribution, it could be due to *E. coli*

persistence and replication within the soil. From the trends seen in Fig. 2.4 fresh manure (less than 14 days old) may be the cause for the higher *E. coli* concentrations at stocked sites, while persistent soil *E. coli* may be the source of the high background concentrations at the destocked and non-grazed sites. Some watershed studies have used total suspended solids (TSS) as an indicator of stormwater quality (Charbeneau and Barret, 1988), or have correlated and interpolated colony forming units (CFU) per grams of eroded soil to estimate the total fecal load (Walker et al., 1990). However, in one study, Wagner (2011) observed *E. coli* concentrations were not well correlated with turbidity. This indicated turbidity and possibly TSS may not always be an appropriate model for representing bacterial loading, and bacterial cells may not always be transported via soil particle attachment. This underscores the dynamic and difficult nature of modeling fecal indicator organisms in the environment and the need for further research. As Fig. 2.5 suggests, stocking rate had no appreciable effect *E. coli* concentrations after 14 days of being destocked (P=0.19). However, Fig. 2.6 shows while field plots were actively or recently stocked (i.e. within 14 days of being destocked), stocking rate did have an effect on *E. coli* concentrations.

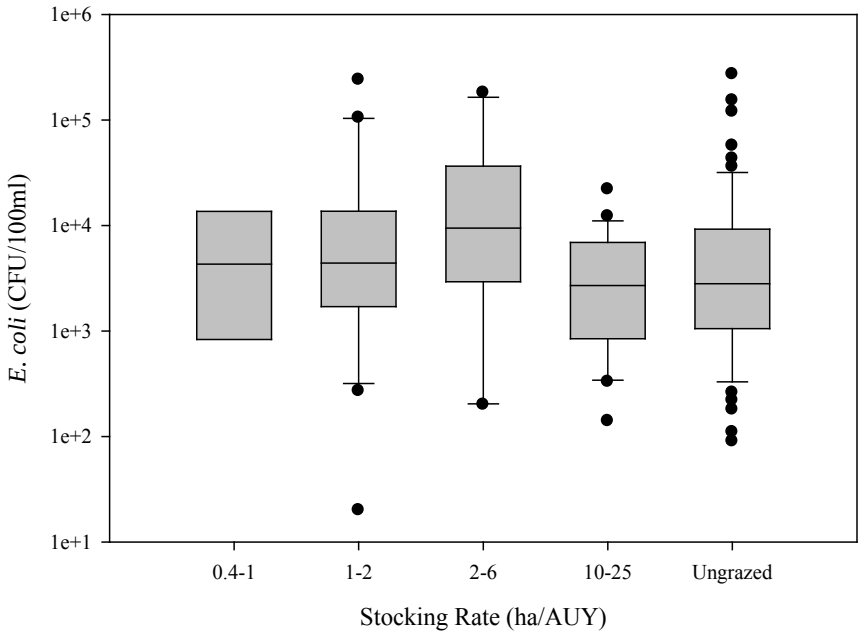

Figure 2.5 *E. coli* concentrations compared between stocking rates. Runoff occurred while field plots were destocked for greater than 14 days. Runoff from ungrazed pastures is included as a reference.

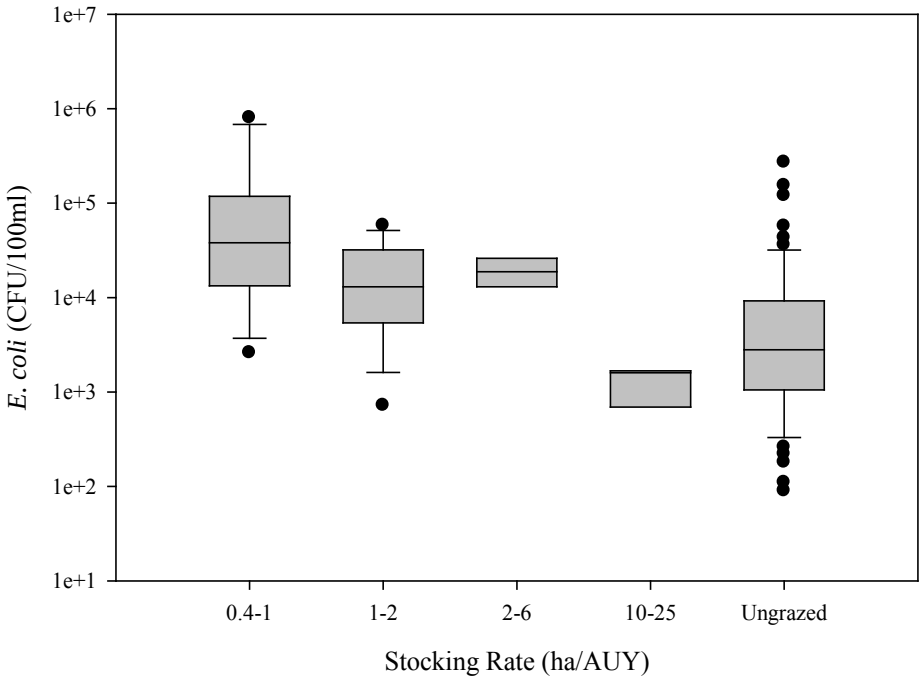

Figure 2.6 Comparison between stocking rates and *E. coli* concentrations in runoff occurring at grazed sites while plots were stocked or within 14 days of being destocked. Runoff from ungrazed pastures is included as a reference.

E. coli concentrations for stocking rates from 0.4 to 1 ha/AUY (1 to 2.5 ac/AUY) and 2 to 6 ha/AUY (4.9 to 14.8 ac/AUY) were significantly higher ($P<0.05$) than the 10 to 25 ha/AUY (24.7 to 61.8 ac/AUY) and non-grazed *E. coli* concentrations. Wagner 2011 also reported *E. coli* concentrations were generally lower for stocking rates lighter than 10 ha/AUY (24.7 ac/AUY). From the results shown in this study, stocking rates heavier than 10 ha/AUY (24.7 ac/AUY) were assumed to increase *E. coli* loading, but only when runoff occurred while sites were stocked or within 14 days of being destocked.

As a means of reducing bacterial loading, Wagner 2011 suggested grazing events be deferred from creek pastures during spring and fall months when rainfall is typically at its highest. This may also help reduce compaction and erosion due to cattle trampling, as well as allow more time for grasses to rest and recover from grazing events.

2.4.2 Location and Site Effects

An overall comparison of the median *E. coli* concentrations from stocked and destocked sites between locations is shown in Fig. 2.7 Results indicated the Riesel location was significantly different from the BCSC site ($P<0.05$). Differences between the Riesel and Welder and the BCSC and Welder locations were considered insignificant ($P>0.05$). The Welder stocked location was determined to be different from the BCSC and Riesel stocked locations ($P<0.05$); however, using the Dunn's Method data analysis, the analysis indicated the dataset was inappropriate for comparing the data between BCSC and Riesel locations. Although the differences in stocked locations are likely due to differences in stocking rate, reasons for the large difference between the destocked BCSC and Riesel locations were not due to stocking rate or time between grazing and runoff events. As such, it is unknown exactly why *E. coli* at non-grazed sites varied so greatly between locations. Watershed characteristics and spatial variability are thought to play a powerful role in the highly variable nature of *E. coli* loading, and are the more likely cause for the differences seen between destocked sites and locations (Harmel et al., 2010). In general, results from Fig. 2.7 were inconclusive in explaining differences in bacterial concentrations between locations.

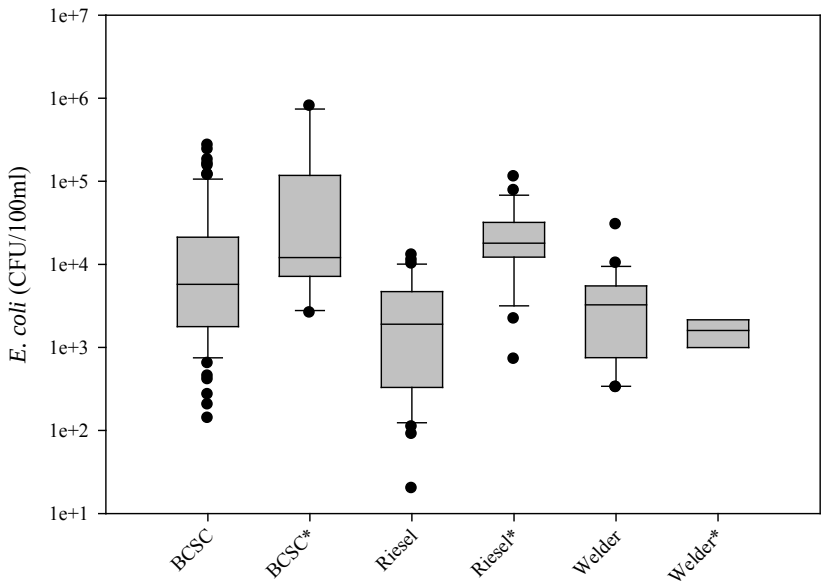

Figure 2.7 Stocked and destocked *E. coli* concentrations compared by locations. Stocked runoff events are denoted with an '*' symbol.

Individual site comparisons shown in Fig. 2.8 yielded more insightful information. Median site values were evaluated collectively across the five year duration. There were no significant differences between the median values at any sites both between sites within locations and sites at other locations. Disregarding stocking rate and the amount of time that had passed between grazing events and runoff events, results seen in Fig. 2.8 indicated differences in bacterial concentrations are not likely due to differences between sites.

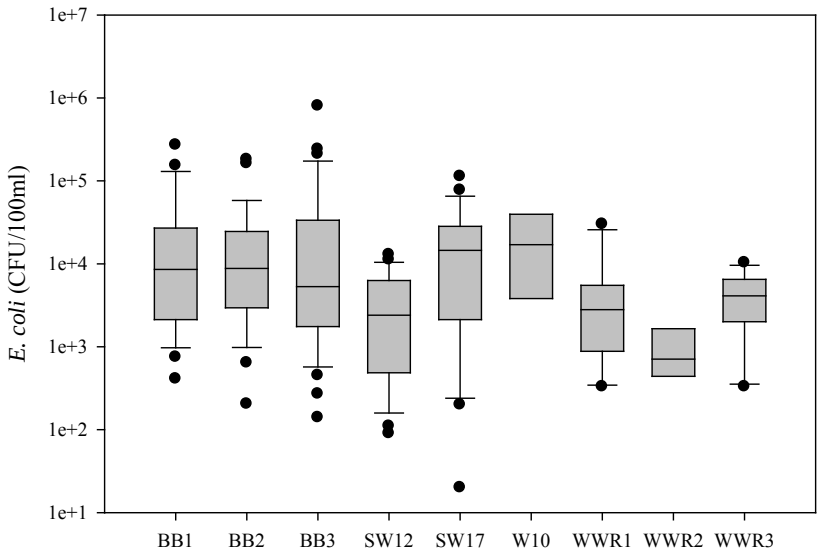

Figure 2.8 Site comparison of *E. coli* concentrations from cumulative years 2008-2012.

Figure 2.9 shows sites BB1, BB2, and SW12 separated by year. The median values of these plots separated by year were analyzed. Sites with less than 3 runoff events per year were excluded from the analysis. Bacterial concentrations from destocked site SW12 in 2009 were determined to be significantly less than site BB1 in 2009 and 2012. Although both BB1 and SW12 were not grazed through the study and despite the runoff events occurring on the same day in most cases, *E. coli* concentrations were significantly lower at SW12 in 2009. This difference may have been caused by bacterial contributions from wildlife.

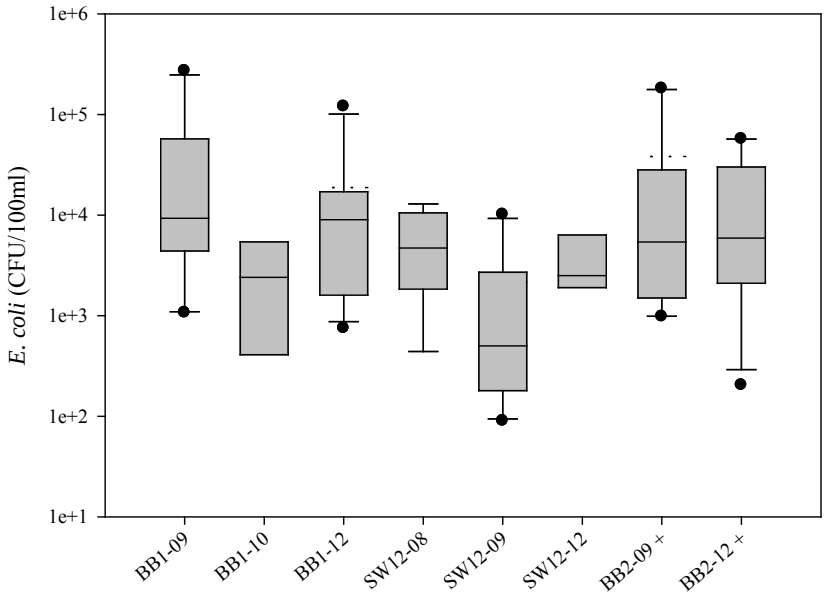

Figure 2.9 *E. coli* concentrations compared between sites BB1, BB2, and SW12, and separated by year. Sites with less than 3 runoff events per year were excluded. Runoff events that occurred on sites while cattle were stocked were excluded from analysis and are denoted with the '+' symbol.

Environmental factors such as drought impeded the ability to replicate approximate field conditions between runoff events as well as limited the total number of runoff events and timing of grazing events. This added to the difficulty of analyzing the relationship between cattle stocking rates and edge-of-field microbial loadings. Environmental, temporal, spatial, and random sampling variability are the more probable causes for any statistical differences between non-grazed or destocked sites; moreover, bacterial load contributions from wildlife or other natural background sources should also be considered. The factors causing bacteria loadings to vary so greatly among non-grazed and destocked sites under relatively similar environmental conditions remain poorly understood. One study observed higher runoff volumes generally correlated with

higher pollutant loading (Charbeneau and Barrett, 1998). However, upon graphing *E. coli* concentrations against runoff volumes (as evident in Fig. 2.10), no clear correlations were observed.

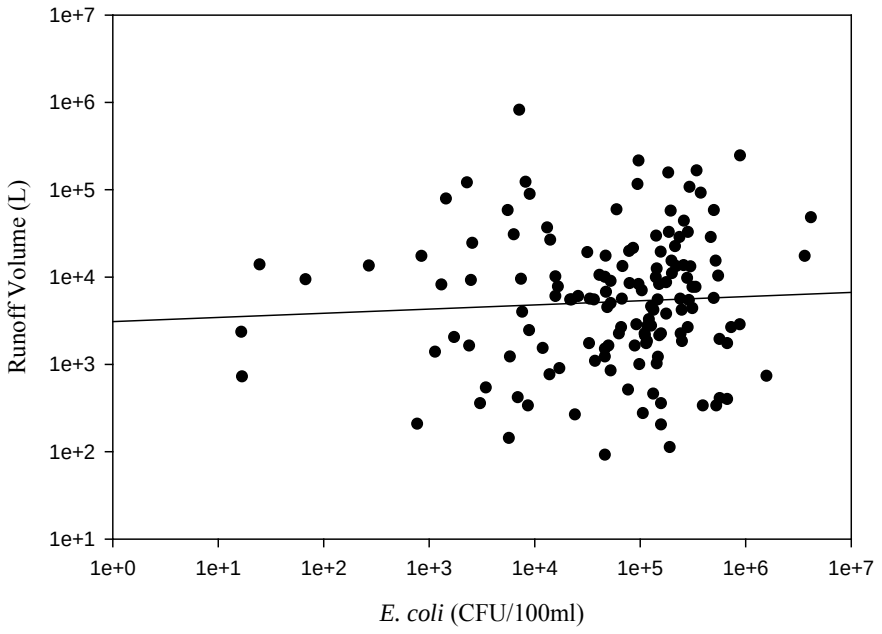

Figure 2.10 Total runoff flow versus *E. coli* concentrations

This was also observed in another study (Harmel et al., 2010), and suggested *E. coli* and other microbial indicators on agricultural grazing-lands may not follow simple empirical models and should be treated uniquely. A significant ($P < 0.0001$) linear correlation was observed between fecal coliform and *E. coli* concentrations as shown in Fig. 2.11 (R^2=0.85). This was expected as *E. coli* are part of the fecal coliform group. *Enterococcus* and *E. coli* were not as strongly correlated ($R^2 = 0.18$, $P = 0.0033$).

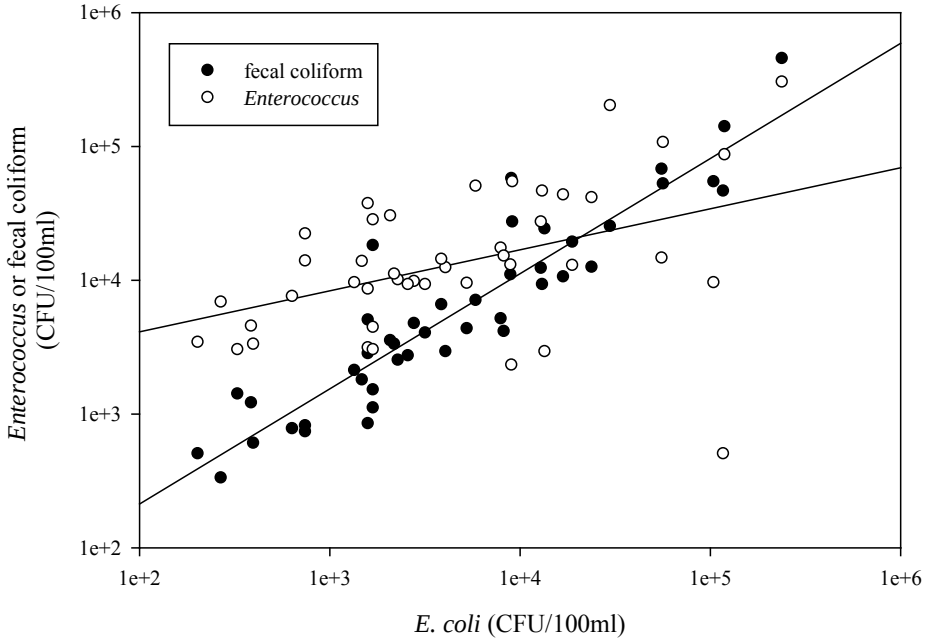

Figure 2.11 *Enterococcus* and fecal coliform concentrations correlated with *E. coli* concentrations

No significant differences were observed between location or sites for either fecal coliform bacteria (P=0.36) in Fig. 2.12 or *Enterococcus* (P=0.182) in Fig. 2.13. Because of the relatively low number of runoff events, it was expected random sampling variability would be large and would not effectively account for treatment effects between sites or location.

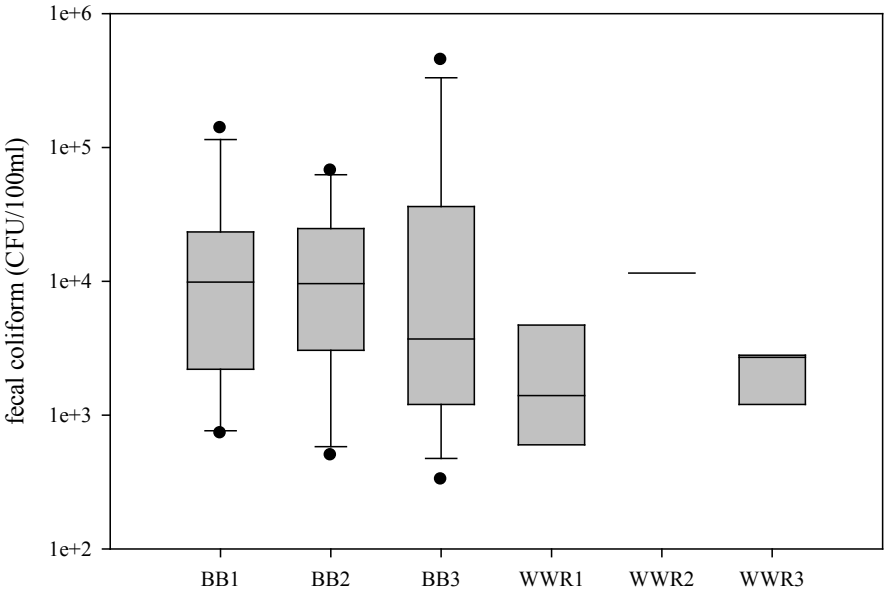

Figure 2.12 Fecal coliform concentrations compared by sites.

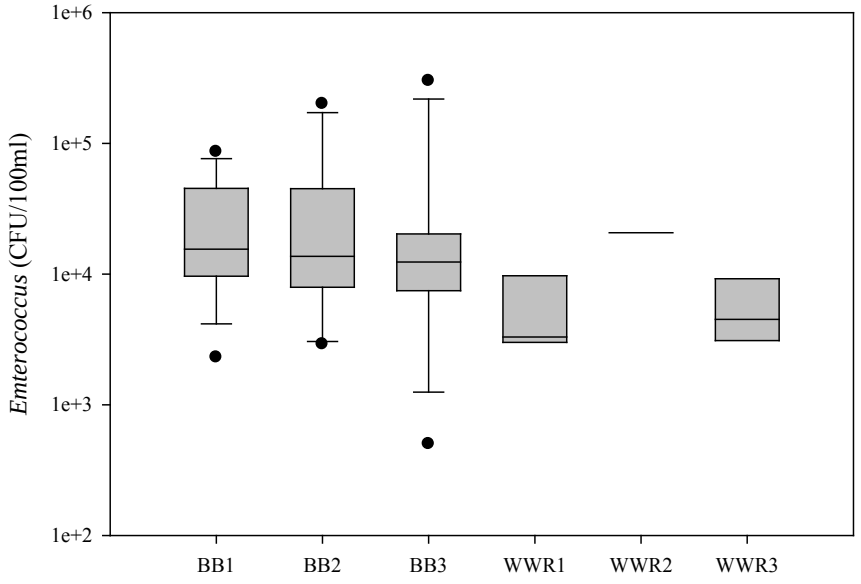

Figure 2.13 *Enterococcus* concentrations compared by sites.

2.4.3 Alternative Shade

GPS trials 1, 2, and 4, as shown in Table 2.2, assessed the effectiveness of a shade pavilion at reducing the amount of time cattle spent in or near the creek. Daily minimum and maximum temperatures, relative humidity, and solar radiation values for each trial are provided in Appendix C-1 through C-5.

Table 2.2 Start and end dates of GPS trials

	Start	BMP Implemented	End
Trial 1	7-Oct-10	15-Oct-10	27-Oct-10
Trial 2	26-May-11	6-Jun-11	18-Jun-11
Trial 3	18-Nov-11	18-Nov-11	9-Dec-11
Trial 4	28-Mar-12	7-Apr-12	18-Apr-12
Trial 5	26-Apr-12	8-May-12	18-May-12

Contrary to the results of Agouridis et al. (2004), after implementing the shade pavilion, significant reductions in the amount of time cattle spent within the riparian zone were repeatedly observed. On average, the amount of time cattle spent within the riparian zone was reduced by at least 30% following implementation of an alternative shade pavilion. Percent reductions ranged from 31% to 45% (Table 2.3). The average of the collective trials, as calculated from Table 2.4, showed cattle spent an average of 51 min d^{-1} within 8 m of the creek prior to implementing the shade structure. Another 61 min d^{-1} were spent within the distance of 8 to 16 m from the creek. Following BMP implementation, collective min d^{-1} averages were reduced by 10.9 and 22.4 min for the 8 and 16 m riparian buffers respectively.

Table 2.3 Alternative shade data-point totals and percent difference calculations per trial. All data points collected prior to BMP implementation are represented by the acronym 'Pre'. 'Post' represents all data points collected after BMP implementation. '% Diff' represents the percent reduction or increase between 'Pre' and 'Post' periods. The '8m' and '16m' symbols represents buffers 0 to 8 m and 8 to 16 m from the stream or shade pavilion respectively.

	Total Points		Shade Pavilion						Riparian Zone					
			8m			16m			8m			16m		
	Pre	Post	Pre	Post	% Diff	Pre	Post	% Diff	Pre	Post	% Diff	Pre	Post	% Diff
Trial 1	14,382	22,465	38	212		27	88		468	401		640	602	
%			0.26	0.94	257.2	0.19	0.39	108.7	3.25	1.78	-45.1	4.45	2.68	-39.8
Trial 2	17,135	18,543	5	157		4	31		871	966		1,063	787	
%			0.03	0.85	2,801.6	0.02	0.17	616.2	5.08	5.21	2.5	6.20	4.24	-31.6
Trial 4	15,552	20,736	3	24		13	32		370	300		330	247	
%			0.02	0.12	500.0	0.08	0.15	84.6	2.38	1.45	-39.2	2.12	1.19	-43.9
Mean					1,186.2			269.8			-27.3			-38.4

Moreover, the amount of time cattle spent at the site of the shade pavilion increased following implementation of the shade pavilion. Cattle increased the amount of time they spent within the 0 to 8 and 8 to 16 m pavilion buffers by 7.6 and 2 min d^{-1} respectively. This accounts for almost a third of the time reductions seen at the riparian zone, and provides supporting evidence percent reductions seen at the riparian zone are due to the alternative shade structure and not some other cause. The 2 min d^{-1} increase in the 8 to 16 m pavilion buffer suggested cattle will graze the area surrounding the shade pavilion more, allowing more grazing to occur on previously underutilized sections of a pasture. It is likely the other half of the time reductions seen in the riparian zone were distributed across the pasture at further distances from the shade pavilion.

Table 2.4 Comparison of average min d^{-1} calculations pre and post BMP implementation per shade trial. The '8m' and '16m' symbols represents buffers 0 to 8 m and 8 to 16 m from the stream or shade pavilion respectively.

	Shade Pavilion				Riparian Zone			
	8m		16m		8m		16m	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Trial 1	3.8	13.6	2.7	5.6	46.9	25.7	64.1	38.6
Trial 2	0.4	12.2	0.3	2.4	73.2	75.0	89.3	61.1
Trial 4	0.3	1.7	1.2	2.2	34.3	20.8	30.6	17.2
Mean	1.5	9.1	1.4	3.4	51.4	40.5	61.3	39.0

Prior to BMP implementation, cattle spent a higher percentage of time within 0 to 16 m of the creek during Trial 2 (11.3%) compared against Trials 1 (7.7%) and 4 (4.5%). This indicated cattle were more dependent upon the riparian shade during Trial 2. Although Trial 2 percent reductions were smaller during warmer months of May and June, Trial 2 min d^{-1} reductions were larger for the 8 to 16 m riparian buffer than either Trial 1 or 4. Although cattle's dependence on shade will be higher in the hotter months, this indicated an alternative shade pavilion may still effectively reduce the amount of time cattle spend within the riparian zone.

On days cattle used the shade pavilion the most, it was expected the amount of time cattle spent within the riparian zone would be significantly lower. While min d^{-1} increases at the shade pavilion are evident across the average of the trials, interestingly, no clear trends were observed on the daily scale.

On one occasion, following implementation of the shade pavilion, a 2.5% increase was observed in the total amount of time cattle spent within the 8 m riparian

buffer; however, the same trial shows a 31% reduction in the 8 to 16 m riparian buffer. This abnormality may be due to a number of possible factors; however, because no water was flowing within the creek during Trial 1 and 2, the increased usage during Trial 2 is not assumed to be due to accessing water. It is possible that cattle sleeping in the creek bed caused the divergence seen in Trial 2. In fact, on several occasions during Trial 2, the time data corresponding with the GPS location indicated several cows had spent the night within 8 m of the creek. Another possible reason may be due to temperature differences between trials, and an increased dependence upon shade. Trials 1 and 4 were completed in October and April respectively while Trial 2 was completed in the hotter month of May. The data shows cattle used the riparian zone during Trial 2 more than either of the other two trials. Larsen et al., (1994) and Byers (2004) also observed temperature and other atmospheric conditions played a significant role in how cattle use the riparian zone and other watershed features.

Expected percentages of cattle usage were estimated by assuming cattle usage was normally distributed across an area. Surprisingly, cattle used the riparian zone less when water was flowing in the creek as seen in Trial 4 from Table 2.5. It is difficult to interpret exactly why this occurred, or if it would occur again. Some possible suggestions are when the creek is dry, cattle will bed-down, loaf in the shade, or even travel in the creek-bed. Of all the time cattle spent within the creek during Trials 1 and 2, 20% of all the data points were found in the creek-bed beneath one small riparian thicket. From visual observations, it was obvious cattle used the area extensively, as it

was easily accessible and well shaded. It is also likely seasonal differences in temperature had an effect on cattle using the riparian zone.

Table 2.5 Expected percent usage of shade pavilion and riparian buffers compared with actual percent usage per trial. Expected values were calculated to represent cattle pasture utilization for a given area assuming the cattle were normally distributed. Observed values represent both pre and post BMP implementation. The '8m' and '16m' symbols represents buffers 0 to 8 m and 8 to 16 m from the stream or shade pavilion respectively.

	Riparian Zone				Shade Pavilion			
	8 m		16 m		8 m		16 m	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Trial 1	3.25	1.78	4.45	2.68	0.26	0.94	0.19	0.39
Trial 2	5.08	5.21	6.20	4.24	0.03	0.85	0.02	0.17
Trial 4	2.38	1.45	2.12	1.19	0.02	0.12	0.08	0.15
Mean	3.57	2.81	4.26	2.71	0.10	0.64	0.10	0.24
Expected	3.85		3.93		0.05		0.15	

More important, in Table 2.5, is the observation percent usage decreased at the riparian zone and increased at the shade pavilion following BMP implementation. Comparing the observed shade pavilion values against the expected value reveals how drastically an alternative shade pavilion can alter cattle pasture utilization. Following implementation of the BMP in Trial 1, cattle proceeded to use the area within the 8 m shade pavilion buffer, an area representing 0.05% of the total pasture area, almost 1% of the total time spent within the pasture.

Byers (2004) observed cattle rested within the riparian zone between 1.4% and 4.2% between December and March, while between April and November, cattle spent anywhere from 5.3% to 8.1% of their time within the riparian zone. In this study, prior

to BMP implementation, cattle spent close to 7.7% of their time within the riparian zone for the month of October and 4.5% from late March to early April. This falls within the range found in a study conducted in Georgia (Byers 2004). However, for the month of May, cattle spent 11.2% of their time within 16 m of the stream. The largest percent of riparian usage found by Byers (2004) was 8.1%. The difference is likely due to the presence of abundant non-riparian shade in the Byers (2004) pasture configurations. This suggested pastures with little non-riparian shade may see increased usage of riparian shade; potentially causing more riparian and water quality degradation. Following BMP implementation at the McGregor pasture, riparian usage was reduced to 9.45% for the month of May. This suggested non-riparian shade does reduce the amount of time cattle spend within riparian areas. Although a single shade pavilion can reduce cattle dependence upon riparian shade, during hotter months, the abundance of riparian shade may draw cattle into riparian zones more frequently and for longer periods. Strategic placement of multiple shade pavilions may optimize pasture utilization and further minimize cattle dependence on riparian shade. Shade pavilions capable of being disassembled and reassembled or transported may also benefit cattle and other livestock producers using an intensive rotational grazing management method.

2.4.4 Climate and Weather Effects

As temperature, relative humidity, and solar radiation increased, it was expected the time cattle spent in the riparian zone would also increase. While seasonal trends were observed between trials, this trend was not seen on a day-to-day basis within trials. When cattle usage of the riparian zone was correlated against temperature, relative

humidity, and solar radiation, no highly evident trends were observed. Seasonal climate differences between trials as well as changes in forage type and quality are reasonable assumptions as to why cattle used the riparian zone differently between the three trials. However, in the short term, daily temperature, relative humidity, and solar radiation were unable to effectively model this. The inability to correlate these climatic co-factors to time spent within the riparian zone on a daily scale suggested cattle use the riparian zone sporadically and for reasons in addition to shade and water. As mentioned by Ganskopp (2001), although the GPS data performed well during these trials, it is often difficult to distinguish whether cattle are using the riparian zone for its shade, water, forage, or other features.

2.4.5 Alternative Water

The alternative water BMP results are based off of one trial; thus, further research should be conducted, and any conclusions should be qualified as such. From Table 2.6, it is evident the alternative water source was not as effective at decreasing the time cattle spent within the riparian zone as the shade pavilion. The amount of time cattle spent within 8 m of the creek actually increased by 46% after implementing the alternative water source. This was contradictory to the initial expectations of the alternative water BMP. An 18% decrease was observed in the 8 to 16 m riparian buffer, but this decrease is not expected to be due to the alternative water source. This suggested cattle can be strongly drawn to riparian areas for reasons other than water as suggested by others (Clawson, 1993; Godwin and Miner, 1996; Miner et al., 1992; Wagner, 2011).

As reported by Ganskopp (2001), a secondary water source can be an effective tool for modifying cattle distribution within a pasture. Indeed, cattle did use the water trough. In fact, as shown in Table 2.7, there was an average 44 min d^{-1} increase following BMP implementation within 16 m of the trough. Prior to BMP implementation, cattle spent less than 43 min d^{-1} within the 0 to 8 m riparian buffer. The difference could have easily accounted for a 100% decrease in time spent within the 0 to 8 m riparian buffer. Nevertheless, as the results show, cattle were still drawn within the 0 to 8 m riparian buffer.

Table 2.6 Alternative water data-point totals and percent difference calculations per trial. All data points collected prior to BMP implementation are represented by the acronym 'Pre'. 'Post' represents all data points collected after BMP implementation. '% Diff' represents the percent reduction or increase between 'Pre' and 'Post' periods. The '8m' and '16m' symbols represents buffers 0 to 8 m and 8 to 16 m from the stream or shade pavilion respectively.

	Total Points		Riparian Zone						Water Trough					
			8m			16m			8m			16m		
	Pre	Post	Pre	Post	% Diff	Pre	Post	% Diff	Pre	Post	% Diff	Pre	Post	% Diff
Trial 5	19,008	17,280	564	750		808	622		8	304		13	245	
%			2.97	4.34	46.3	4.25	3.60	-18.1	0.04	1.76	4,080.0	0.07	1.42	1,973.1

Table 2.7 Min d^{-1} differences between pre and post BMP implementation at riparian and water trough locations. The '8m' and '16m' symbols represents buffers 0 to 8 m and 8 to 16 m from the stream or shade pavilion respectively.

	Riparian Zone				Water Trough			
	8 m		16 m		8 m		16 m	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Trial 5	42.7	62.5	61.2	51.8	0.61	25.3	0.98	20.4

It is unclear exactly why a 46% increase was observed within the 0 to 8 m riparian buffer following BMP implementation. Similar studies have observed 51% to 85% reductions in the amount of time cattle spend within or near a stream (Clawson, 1993; Godwin and Miner, 1996; Miner et al., 1992; Sheffield et al., 1997; Wagner, 2011). Wagner (2011) reported having little riparian or non-riparian shade at the study site and cited it as the most probable reason for the increased effectiveness of the alternative water source. During the post BMP period, water was provided both in the creek and at the alternative water trough. Cattle used the alternative water source; although, it did not reduce the amount of time spent in the riparian zone. Because the cattle used the water trough, the increased time spent within 8 m of the creek is not believed to be caused by an increased dependence upon creek water. During the second shade trial, a 2.5% increase was observed within the 8 m riparian zone following BMP implementation. This too was unexpected, but was not due to any attraction to water because the creek was dry. It is possible the cause of the increased riparian usage seen in Trial 5 was due to cattle sleeping within 8 m of the creek at night. However, the GPS data did not collect the time data for Trial 5; thus, making it impossible to confirm or deny this supposition. Another possible reason would be due to cattle wading within the stream for its cooling effects or to avoid insects such as heel-flies. Although this was also unlikely because the creek had few pools deep enough to provide these benefits. Furthermore, this does not explain the large increase in time; as these effects would most likely be observed throughout the entire trial and not solely during the post BMP

implementation period. The most reasonable explanation for the large increase would be an increased dependence upon riparian shade.

Table 2.8 shows the expected and percent usages for certain pasture and watershed features. Expected percent values were determined by assuming cattle position was normally distributed across an area. Thus, the expected percent usage was the same as the percent area of a certain watershed feature. As seen in Table 2.8, observed values were closely related to the expected values. Following implementation of an alternative water source, percent usage increased from 0.04% to 1.76% and 0.07% to 1.42% for the 0 to 8 and 8 to 16 m trough buffers respectively. Compared to the expected percent usage, this resulted in a 35 and 9 fold increase from the time cattle were expected to spend at the 0 to 8 and 8 to 16 m trough buffers respectively, and it indicated water strongly influences cattle position.

Table 2.8. Expected percent usage of water trough and riparian buffers compared with actual percent usage per trial. Expected values were calculated to represent cattle pasture utilization for a given area assuming the cattle were normally distributed. Observed values are represented both pre and post BMP implementation.

	Riparian Zone				Water Trough			
	8 m		16 m		8 m		16 m	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Trial 5	2.97	4.34	4.25	3.6	0.04	1.76	0.07	1.42
Expected	3.85		3.93		0.05		0.15	

The failure to decrease the amount of time cattle spent within the riparian zone indicated shade, not water, may be the largest driving factor for the amount of time cattle

spend within the riparian zone. Similarly, the alternative shade BMP would be the most appropriate BMP for sites where riparian shade is the primary source of shade. Wagner (2011) commented the amount of time cattle spend in or near a stream varied substantially between similar studies and also attributed the differences in results to site-specific differences in watershed features. The results of this study underscore the importance of implementing water-quality BMPs on a site specific basis, as the alternative water BMP did not reduce the amount of time spent within the riparian zone as effectively. Although it was not tested in this study due to time and drought induced restraints, combining alternative shade and water together would likely have improved effectiveness at reducing the amount of time cattle spend within the riparian zone and improving pasture utilization.

Severe drought conditions impeded the ability to repeat GPS trials due to lack of forage and changes in the day-to-day ranch management schedule and pasture rotations. Drought conditions also limited flow in the creek making scheduling alternative water trials even more difficult.

2.4.6 Preliminary Rip-rap Trial Results

Smaller rip-rap, 10 to 20cm (4 to 8 in) diameter, had moderate to limited effectiveness at detouring cattle from the trough treated with rip-rap, $T_{rip\text{-}rap.}$ With smaller rip-rap, cattle trough preference seemed to be correlated to the cow's proximity and orientation to the trough, available room at the trough, and pecking order. On one occasion, 7 cows watered at $T_{rip\text{-}rap}$ while only 2 cows watered from $T_{control}$. Calves were more frequently observed loitering around $T_{rip\text{-}rap}$. Although both young and mature

cows watered from both $T_{rip\text{-}rap}$ and $T_{control}$, the younger and less dominant cows tended to water more from $T_{rip\text{-}rap}$ than the older and more dominant cattle. With some exceptions, cattle showed some slight hesitation prior to walking over the smaller rip-rap. On multiple occasions, some cows would walk around the perimeter of the rip-rap before approaching $T_{rip\text{-}rap}$ from the most level and compacted walking path. This provided some evidence rip-rap can work to modify cattle walking paths, and it also showed evidence about cattle's preference for walking over stable and more even ground as mentioned by Miner et al., (1992). Some differences in the amount of time cattle spent loitering near the troughs were observed between $T_{rip\text{-}rap}$ and $T_{control}$; however, these differences seemed nominal, and varied between trials. When cattle crowded $T_{control}$, the remaining cattle would drink from $T_{rip\text{-}rap}$. At other times, the more dominant cows would push their way to $T_{control;}$ causing the less dominate cows to wait or move to $T_{rip\text{-}rap}$ which they often did.

Although the cattle did not always respond as desired to the smaller rip-rap treatment; overall, more cattle watered from $T_{control}$ throughout the preliminary trials than from $T_{rip\text{-}rap}$ (Table 2.9). The increased usage from $T_{control}$ suggested cattle did respond to the smaller rip-rap treatment albeit delayed and with limited effectiveness. These preliminary demonstrations showed smaller diameter rip-rap may not be an effective tool for modifying cattle behavior. Because $T_{rip\text{-}rap}$ and $T_{control}$ were the only two water sources available to the cattle, the effectiveness of the rip-rap may have been minimized by cattle's strong need for the water. Smaller rip-rap may be more effective at modifying

cattle walking trails rather than attempting to exclude them from a water, shade, or forage source altogether.

Table 2.9 Number of cows that watered at $T_{control}$ and $T_{rip\text{-}rap}$ throughout the day. Smaller, 10 to 20 cm (4 to 8 in) diameter, rip-rap was used during this preliminary trial. A total of 16 cows were used in this trial.

Time	$T_{Rip\text{-}Rap}$	$T_{Control}$
10:45*	7	2
14:00+	10	10
16:10+	4	16
18:50+	6	16

* Not all cattle watered
+Some cows watered from both troughs

The larger rip-rap, 20 to 40 cm (8 to 16 in) diameter, was observed to be highly effective. Cattle trough preference was strongly altered by the larger rip-rap treatment. Cattle showed extreme hesitation at crossing the larger rip-rap. On one occasion, one cow attempted to cross the rip-rap, but turned around before reaching the trough. On several occasions no less than a dozen cows stood at the perimeter of the rip-rap looking for long periods at the water in $T_{rip\text{-}rap}$ before eventually watering from $T_{control}$. Even when there was no available room at $T_{control}$, cattle did not water from $T_{rip\text{-}rap}$. Loitering did not occur at $T_{rip\text{-}rap}$ once the larger rip-rap was implemented. Similar to the smaller rip-rap, the detouring effectiveness of rip-rap decreased with younger calves. On one occasion, two mature cows laboriously crossed the rip-rap to water from $T_{rip\text{-}rap}$. Although the majority of the results show cattle are less likely to cross the rip-rap

treatment, this indicated cattle are capable of crossing the larger rip-rap if they wish or must. Although neither rip-rap treatment totally excluded cattle, there was a major difference in the effectiveness between the small and large rip-rap treatments for both young and old cows alike. By increasing the size of the rip-rap, the treated area became more irregular and uneven, and cows had to exert a larger amount of effort to cross. The larger rip-rap may be the most appropriate cattle deterrent especially when there is a stronger need to limit cattle's access to a particular location or resource such as for riparian zone vegetation reestablishment.

Serious consideration should be made before implementing rip-rap as a water-quality BMP. Although the larger rip-rap showed a greater ability to deter cattle, it was not 100% effective, and cattle crossing rip-rap could potentially injure themselves. For this reason, cattle producers looking to implement rip-rap as a water-quality BMP should also consider the appropriateness of rip-rap placement and the potential for injury of the cattle should they attempt to crossover the rip-rap.

2.4.7 Rip-Rap GPS Trials

Rip-rap results from the GPS trials were largely inconclusive. Due to several study limitations including dry weather conditions, this study failed to fully assess how cattle responded to the rip-rap treatment in a pasture setting. The location of the rip-rap was chosen based on the high density of GPS points within four streamblocks (12 through 15). This site was assumed to be a location at which cattle crossed the stream

most frequently; however, upon further review, the true reason for the high density of points was due to cattle loafing in the dry streambed beneath shade. It was difficult to accurately assess where the cattle had crossed the stream even when interpolating between two GPS points taken on either side of the creek. The time intervals between GPS points were too long to accurately characterize where the cows crossed the stream.

Results of the study are represented in Fig. 2.14. Of the time cattle spent within the streambed during trials 1 and 2, cattle spent over 12% of their time within streamblocks 12 through 15. After the rip-rap treatment was implemented, cattle spent a lower percentage of their time within streamblocks 12 through 15; however, cattle collectively spent less time within the stream during trials 3 and 4, so it is likely that cattle were influenced by other detouring factors such as temperature or the presence of water in the creek, as the creek was often dry during the study due to the extended drought, and they tended to loaf in the streambed where shade was present.

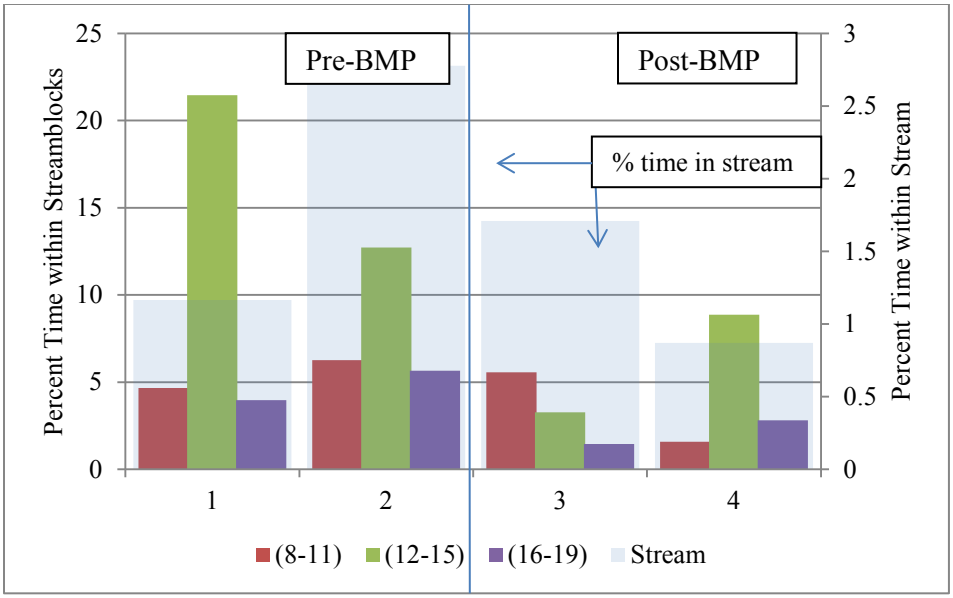

Figure 2.14 Rip-rap results for Trials 1 through 4 at streamblocks 12 through 15. Light blue bars represent the total percent of time cattle spent within the streambed, and are represented by the right y-axis. Red, green, and purple bars represent the percent time cattle spent within their respective streamblocks relative to the total percent time cattle spent within the streambed, and are represented by the left y-axis.

The rip-rap was reconfigured to streamblocks 1 through 4 during Trial 5. The results of the trial are shown in Figure 2.15. Cattle spent a low percent of their time in the stream during the first half of Trial 5; however, of the time they did spend in the creek, a significant portion of it was spent within streamblocks 1 through 4. During the second half of the trial, cattle spent more of their time within the streambed, but a slightly lower percentage at streamblocks 1 through 4. In reality, cattle spent more time within streamblocks 1 through 4 after rip-rap was implemented. One possible explanation of this was that in Trial 5, rip-rap was only implemented on the higher bank where a well define cattle trail existed. The increase in the total amount of time the

cattle spent within streamblocks 1 through 4 may be due to the cattle approaching the creek from the untreated side and having to find an alternative route around the rip-rap.

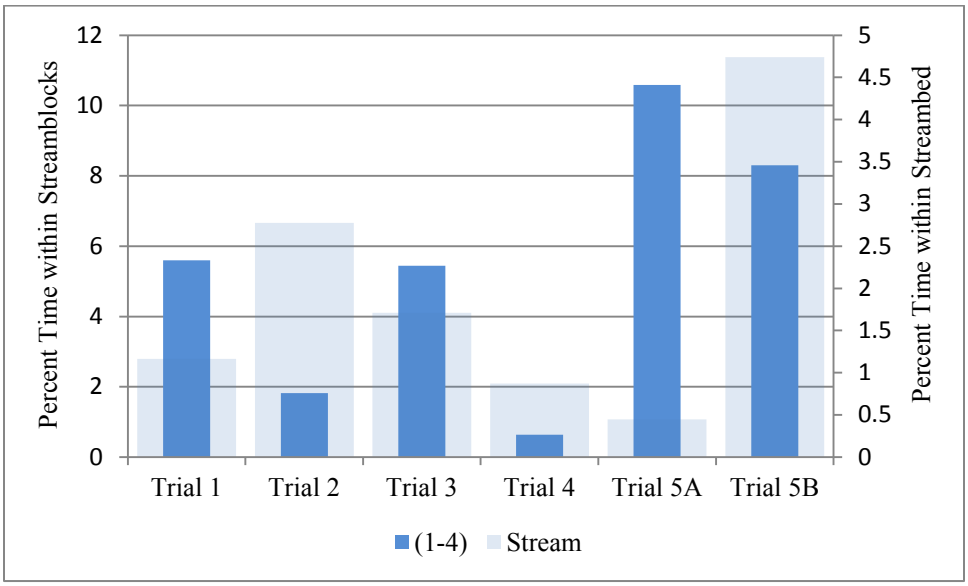

Figure 2.15 Rip-rap results for Trials 1 through 5 at streamblocks 1 through 4. Light blue bars represent the total percent of time cattle spent within the streambed, and are represented by the right y-axis. Dark blue bars represent the percent time cattle spent within streamblocks 1 through 4 relative to the total percent time cattle spent within the streambed, and are represented by the left y-axis.

Although the results of this study were largely inconclusive, it does not necessarily preclude rip-rap as a successful water quality BMP. Future rip-rap studies should not determine rip-rap placement based on GPS data alone. This is true because it is often difficult to determine the exact reason why the cattle were at a particular point whether it be for shade, water, rest, forage, or a stream crossing point. Secondly, producers utilizing these BMPs will rely on visually observed crossing sites rather than

GPS data. Future structural BMP studies may use satellite imagery as a tool for analyzing how livestock or wildlife interact with structural BMPs.

2.5 Conclusions

2.5.1 Non-Structural BMPs

No significant differences in *E. coli* concentrations were observed between *E. coli* concentrations in runoff from heavily stocked, moderately stocked, or non-grazed pastures when pastures had been destocked for greater than 14 days. However, while pastures were actively stocked or within 14 days of being destocked, *E. coli* concentrations were significantly higher than destocked pastures. Thus, the null hypothesis was rejected because bacterial concentrations in runoff did vary significantly between different stocking rates, but only when sites were stocked when runoff occurred. *E. coli*, *Enterococci*, and fecal coliform concentrations varied greatly between runoff events even when no apparent differences in stocking or timing treatments existed. Background *E. coli* concentrations from non-grazed pastures were also very high and varied greatly between runoff events.

2.5.2 Structural BMPs

Strategic placement of a shade structure reduced cattle's dependence on riparian shade, so the null hypothesis was rejected. Both the alternative shade and water BMPs helped improve pasture utilization. The alternative water BMP did not reduce the amount of time cattle spent within the riparian zone for this particular study, so the null hypothesis was accepted. However, for the alternative water study, drought conditions limited the GPS study to a single trial. Cattle used the riparian zone in this pasture primarily for shade, as the alternative water BMP was not effective at decreasing time cattle spent within the riparian zone despite the secondary water source being well utilized. Results from the riparian rip-rap trials were inconclusive, and the null hypothesis was accepted; however, preliminary rip-rap trials showed larger, 20 to 40 cm (8 to 16 in) diameter, rip-rap was highly effective at modifying cattle trough preference.

CHAPTER III

VARIABILITY WITHIN SEQUENCES DETECTED BY BACTEROIDES BST MOLECULAR MARKERS

3.1 Introduction

One of the largest difficulties with attaining non-impairment levels of bacteria in state and national waterways has been accurately assigning pollution to a specific source. The dynamic nature of pollutant transport and fate can easily frustrate efforts to improve water quality. Without clarification of the source, certain BMPs may be postulated as the solution to achieving microbial water-quality standards (WQS) even if the BMP does not effectively address the underlying pollution problem. By using bacterial source tracking (BST) methods, specific species can be targeted as the main source(s) of fecal pollution, and BMPs can be applied more appropriately. Several BST methods have emerged providing a more efficient approach to identifying the source of bacterial impairments in our nation's waterways.

Advances in library-independent, culture-independent genotypic methods have led to the discovery of *Bacteroides* as a valuable BST molecular marker. *Bacteroides* are a genus of gram-negative, anaerobic, enteric bacteria found to comprise a significant portion of the total fecal flora present in many species' digestive tracts including humans (Holdeman et al., 1976) and bovine (Dowd et al., 2008). *Bacteroides* are used as a BST marker because of their ubiquitous presence in the intestinal tract of many domestic and feral animal species, their host-specificity (Bernhard and Field, 2000), and their short persistence outside of host (Anderson et al., 2005; Kreader, 1998).

Host-specific sequences from *Bacteroidales* and *Bacteroides* spp. have been identified for many species (Bernhard and Field, 2000; Dick et al., 2005; Fogarty and Voytek, 2005). However, only a few *Bacteroides*-based molecular markers have been developed and tested including human, bovine (Bernhard and Field, 2000; Layton et al., 2006), canine (Kildare et al., 2007), and swine species (Ufnar et al., 2007). Some of these molecular assays have been used in field studies to identify and quantify the amount of fecal pollution from specific sources; and, as with most newly developed methods, there have been a few technical drawbacks discovered along the way.

A recent study (Wagner, 2011) using the universal (AllBac) and bovine (BoBac) molecular markers developed by Layton et al. (2006) suggested there may be geographic variability among *Bacteroides* populations that might account for some of the differing results found in the study. The study compared *E. coli* concentrations against AllBac and BoBac marker concentrations in three different locations in Texas. Results varied between sites. At the site where the feces were collected for gene copy curve development, the markers worked well (Wagner, 2011). At the other two locations, no correlation was observed between markers and *E. coli*. This suggested potential geographical variability within sequences detected by the AllBac and BoBac molecular markers. Similarly, it suggested the gene copy curve created from feces collected at one location to quantify environmental samples may not accurately represent *Bacteroides* populations at other locations. As enteric bacterial populations are highly adaptive (Gordon and FitzGibbon, 1999), it is not unreasonable to believe DNA variations occur

within *Bacteroides* populations due to differences in diet, geography, and other environmental stressors.

The AllBac and BoBac molecular markers were derived from the more conserved regions of the 16S rRNA gene of the *Bacteroides* genome (Layton et al., 2006). The primers and probe should anneal along these regions; however, it is possible for the primers and probe to anneal even when some base-pairs are mismatched. It was suggested by Wagner (2011) there may be significant mismatches due to geographic variability causing the varied results seen in the study. If significant mismatches occur within a *Bacteroides* strain, and if that strain is used to create the gene copy curve, then the resulting samples measured by the gene copy curve will be skewed. This is because qPCR assays are not absolute in their quantification; they only quantify a sample relative to the gene copy curve, or standard curve, used. If the standard curve is inaccurate, the samples will also be quantified incorrectly.

In a study testing for cross-amplification between cattle and avian species, the BoBac marker was detected in both avian and bovine samples (Layton et al., 2006); although, the false-positive avian sequence had several mismatches along the annealing regions. This caused the avian gene-copy curve to be extended from the target bovine gene-copy curve. This example of cross-amplification between avian and bovine samples exposes the difficulty of finding an adequate molecular marker, and also raises questions as to the homogeneity of the *Bacteroides* populations within bovine fecal samples. If mismatches occur among bovine samples when using the AllBac and BoBac

assays, qPCR results will be skewed causing the total amount of in-stream fecal pollution to be under or over estimated.

3.2 Objectives

The over-arching goal of this study was to help reduce misrepresentations of bovine fecal pollution in environmental samples by identifying DNA variances that could affect the AllBac and BoBac assays. Specific objectives included the following:

- Determine if base-pair mismatches occur when using the AllBac and BoBac assays on multiple bovine samples.
- Determine if mismatches significantly affect qPCR efficiencies.
- Evaluate if developing gene copy curves from local fecal samples can improve correlations between *E. coli* and AllBac and BoBac molecular markers.

H_0: Base-pair mismatches do not occur, qPCR efficiencies are not significantly different between environmental samples, and correlations between *E. coli* and AllBac and BoBac molecular markers do not improve with locally developed gene copy curves.

H_1: Base-pair mismatches do occur, qPCR efficiencies are significantly different between environmental samples, and correlations between *E. coli* and AllBac and BoBac molecular markers improve with locally developed gene copy curves.

3.3 Methods

3.3.1 Previous Research and Methods

This study used many of the same runoff samples used in Wagner et al. 2011 including 13 DNA extracted runoff samples and the original standard (Std_0) used for DNA quantification. The standard used to create the original gene-copy curve was created from a fresh bovine fecal deposit from a pasture grazed cow at the Texas A&M University, O. D. Butler, Jr. Animal Science Teaching, Research, and Extension Complex (ASTREC) five miles west of College Station, Texas (Wagner, 2011). Storm water runoff samples were collected from grazed and non-grazed pasture sites at the USDA Agriculture Research Service (ARS) in Riesel, Texas from March of 2008 to February of 2010 (Wagner, 2011). Each runoff sample was enumerated for *E. coli* using EPA Method 1603 (EPA, 2003). Following enumeration, qPCR assays were performed on runoff samples using AllBac and BoBac molecular markers.

3.3.2 Sample Collection, Storage, and Enumeration

The Riesel sampling site was selected as the site of preference because previous data at this site showed the highest variability between *E. coli* concentrations and copy numbers of AllBac and BoBac markers (Wagner, 2011). Twelve fresh bovine fecal samples were collected from ARS in Riesel. Duplicate samples were collected aseptically in sterile 5 mL fecal-collection tubes. Samples were preserved on ice and transported to the Soil and Aquatic Microbiology Laboratory (SAML) in College Station, Texas. Upon arrival, each primary sample was enumerated for *E. coli* using EPA Method 1603, and the duplicate sample stored at -80°F for future qPCR analysis.

3.3.3 Creation of Standards

Samples with detectable levels of *E. coli* were chosen to be used as standards. Frozen samples were thawed, and genomic DNA was then extracted from one gram of fecal material. DNA was extracted using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA), and further purified using illustraMicroSpin S-400 HR Columns (GE Healthcare, UK). Periodically throughout the extraction and purification steps, the total amount of nucleic acid was estimated using a NanoDrop ND-1000 UV spectrometer (NanoDrop Technologies, Wilmington, DE) to confirm the presence of product DNA.

The 16S region of *Bacteroides* DNA was selectively amplified from the genomic DNA using universal Bac primers (32-F and 708-R) (Layton et al., 2006). Endpoint PCR amplification was performed with an Eppendorf thermocycler (Hamburg, Germany). Initial PCR amplification was performed using 50 µl reactions. Genomic DNA was amplified using 25 µl of Failsafe A buffer (Epicentre Biotechnologies, Madison, WI), 30 pmol of primers (32-F and 708-R), 2.5U of Amplitaq Gold (Roche Molecular Systems, Inc, Pleasanton, CA) and the remaining volume with DNase/RNase-free distilled water. The temperature sequence was set at 10 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 60 seconds at 53°C, and 60 seconds at 72°C. A final extension was included for 10 minutes at 72°C (Bernhard and Field, 2000; Field et al., 2003). The *Bacteroides* and template DNA was then separated using gel electrophoresis with ethidium bromide as a DNA intercalator. The amplified *Bacteroides* DNA was extracted from the gel at around 700 bp, and then gel purified using a QIAquick Gel

Extraction Kit (QIAGEN, Valencia, CA). The amount of nucleic acid was again estimated with the NanoDrop spectrometer following the gel extraction step.

The purified *Bacteroides* DNA was ligated into plasmids and inserted into competent *E. coli* cells using QIAGEN EZ Competent Cells. To verify the presence of the insert, *E. coli* cells underwent blue-white screening by being plated onto X-gal treated Luria-Bertani (LB) plates and incubated at 37˚C for 18 hours. Confirmed colonies were plated onto kanamycin treated LB media and incubated at 37°C for 18 hours. For each sample, two positive colonies were pulled from the LB plates and stored at -80°C in glycerol stock. The plasmids containing the *Bacteroides* insert were then extracted from the *E. coli*, quantified, and stored at -20°C. To create standards, the DNA were first estimated using the NanoDrop spectrometer and then quantified using the Quant-It™ Picogreen® assay (Invitrogen) assay. Five standard solutions were created for each sample ranging from 10^{-2} to 10^{-6} ng/µl.

3.3.4 Creating Gene Copy Curves

Quantitative PCR assays were performed on stored runoff samples with Std_0 and the newly created Riesel standards as the reference standards. Gene-copy curves were compared among Std_0 and the Riesel standards, and the 13 stored runoff samples were analyzed against the most divergent standards. Assays were completed for standards using both AllBac and BoBac primers and probes. Assays were completed using 20 µl reactions per well. Each reaction contained 2X QuantiTect Probe PCR Master Mix (QIAGEN, Valencia, CA), 15 µM of both forward and reverse primers for either AllBac

or BoBac assay, 5 pmol probe, 4 µl of template DNA, and the remaining volume was filled with DNase-free DI water. Negative controls include 4 µl of DI water instead of template DNA, and spikes were also included as positive controls using 1 µl of standard RSL2.2 and 3 µl of a runoff sample of a known initial concentration. The temperature sequence was held at 50°C for 2 min, followed by 95°C for 10 min, and 50 cycles of 95°C for 30 seconds, 57°C (BoBac assay) or 60°C (AllBac assay) for 45 seconds, and final extension at 72°C for 60 seconds (Layton et al. 2006). Standards, unknowns, spikes, and no-template/control (NTC) were all processed in triplicate. Once the cycle threshold (CT) values were obtained, the cycle number was graphed against the initial copies/µl. A best-fit, log-transformed line was established for each standard. This line was used to calculate the approximate number of copies in a runoff sample.

3.3.5 Plasmid DNA Sequencing

Prior to sequencing, *E. coli*, containing plasmids with the *Bacteroides* insert, were cultured in ampicillin-containing liquid LB broth. The plasmids were then extracted using a Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The Std_0 and the seven Riesel standards were sequenced using Sanger sequencing methods at the Veterinary Pathobiology DNA Technologies Core Lab in College Station, Texas. Samples were sequenced in both the forward and reverse directions using primers M13F-20 and M13R. Sequenced DNA was aligned using BioEdit, and the primer and probe regions were compared to check for mismatches. A phylogenetic tree was created by combining the Riesel and Std_0 sequences with GenBank sequences used

in the Layton (2006) study. The phylogenetic tree was created using the neighbor-joining command with MEGA version 4 (Tamura, Dudly, Nei, and Kumar 2007).

3.3.6 Statistical Analysis

E. coli concentrations were correlated against estimated AllBac and BoBac concentrations using various standard curves to determine if the R^2 values significantly changed from one standard to the next. *E. coli,* AllBac, and BoBac concentrations were all $\log_{10}$ transformed prior to correlating. All comparisons were analyzed using Sigma Plot statistical software. AllBac and BoBac runoff concentration estimations were $\log_{10}$ transformed and compared using an analysis of variance. The level of significance was set at an alpha value of 0.05. All means with a statistical significance smaller than 5% ($P<0.05$) were determined to be significantly different.

3.4 Results

As expected, the results of this study showed mismatches did occur within the BoBac molecular assays (see Appendix D). Within the Riesel sequences, mismatches were observed along the BoBac primer/probe regions; however, no mismatches were found within the AllBac primer/probe regions. Because of the poorer annealing and extending abilities of sequences with more mismatches, PCR reactions for sequences with mismatches did not amplify as efficiently and took longer to reach the cycle threshold value. Thus, when initial AllBac and BoBac concentrations were estimated for runoff samples using different standard curves, the concentrations were found to vary significantly between standards ($P < 0.05$) (Fig. 3.1 and Fig. 3.2). Standard curves of sequences with more mismatches end up being extended away from the origin and from

other sequences with fewer mismatches. The only difference between the DNA in the Std_0 and Wagner (2011) values is the time at which the assays were taken. The Wagner (2011) values were one to two orders of magnitude higher than Std_0 values, as seen in Tables 3.1 and 3.2. This difference is assumed to be due to DNA degradation over time. AllBac and BoBac estimations for runoff samples varied by up to three orders of magnitude between the most divergent standard curves as seen in Tables 3.1 and 3.2.

Table 3.1 AllBac runoff pollution load estimations compared using three standard curves

Runoff Sample	RSL 2.2	RSL 3.2	Std_0	Wagner (2011)
6	1.36E+01	3.82E+00	1.05E+01	8.50E+02
9	1.52E+01	4.22E+00	1.17E+01	8.40E+02
21	2.99E+01	7.87E+00	2.28E+01	7.46E+02
22	4.98E+03	8.64E+02	3.55E+03	2.39E+04
100	1.11E+03	3.45E+02	1.47E+03	1.30E+04
114	6.66E+03	2.03E+03	8.72E+03	9.16E+04
119	2.99E+02	9.47E+01	4.00E+02	1.07E+04
120	5.22E+00	1.59E+00	4.09E+00	
121	2.53E+01	8.28E+00	3.44E+01	8.92E+02
122	4.58E+03	1.40E+03	6.02E+03	1.58E+05
123	1.63E+03	3.10E+02	1.18E+03	1.03E+04
124	7.05E+01	1.73E+01	5.33E+01	2.96E+03
125	1.13E+02	2.66E+01	8.45E+01	3.57E+03

Table 3.2 BoBac runoff pollution load estimations compared using three standard curves

Runoff Sample	RSL 2.2	RSL 3.2	Std_0	Wagner (2011)
6	2.51E+02	6.45E-02	1.69E-01	3.75E+00
9	4.28E+02	1.07E-01	2.83E-01	2.26E+00
21	1.72E+02	4.49E-02	1.17E-01	7.04E+00
22	2.36E+02	6.07E-02	1.59E-01	2.89E-01
100	2.02E+05	1.01E+02	2.50E+02	6.95E+03
114	1.12E+06	3.96E+02	1.12E+03	1.08E+04
119	5.50E+04	3.60E+01	8.00E+01	1.44E+03
120	1.07E+01	3.12E-03	7.88E-03	7.71E-01
121	3.37E+01	9.97E-02	1.22E-01	5.11E+00
122	1.95E+06	6.16E+02	1.83E+03	2.20E+04
123	-	-	-	2.39E+01
124	1.90E+04	4.07E+00	1.12E+01	7.78E+01
125	3.27E+04	6.85E+00	1.90E+01	2.41E+00

Standard curves are the gauge by which fecal pollution loads in environmental samples are quantified. When using a sequence with mismatches, the standard curve is shifted away from the origin which causes the pollution load to be overestimated. Similarly, if the sequence used for creating the standard curve has no mismatches, but environmental samples are full of sequences with mismatches, then the pollution load can be underestimated. Initial AllBac and BoBac copies L^{-1} concentrations were estimated for runoff samples by using the calculated line equations from the various standard curves. Standard curve RSL-2 in Fig. 3.2 reveals how mismatches within the BoBac primer/probe regions can extend the curve away from the origin. This results in higher initial copies/L estimations as seen in Tables 3.1 and 3.2.

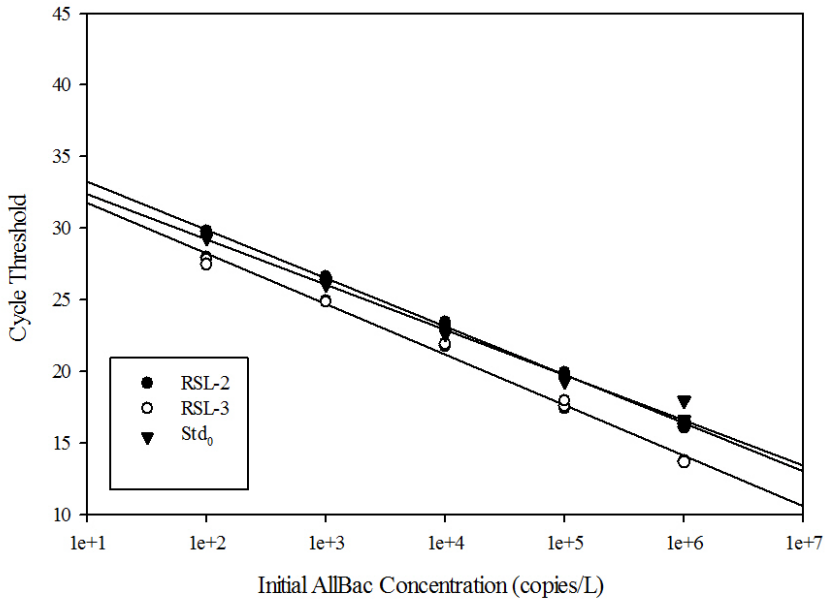

Figure 3.1 Most divergent AllBac standard curves

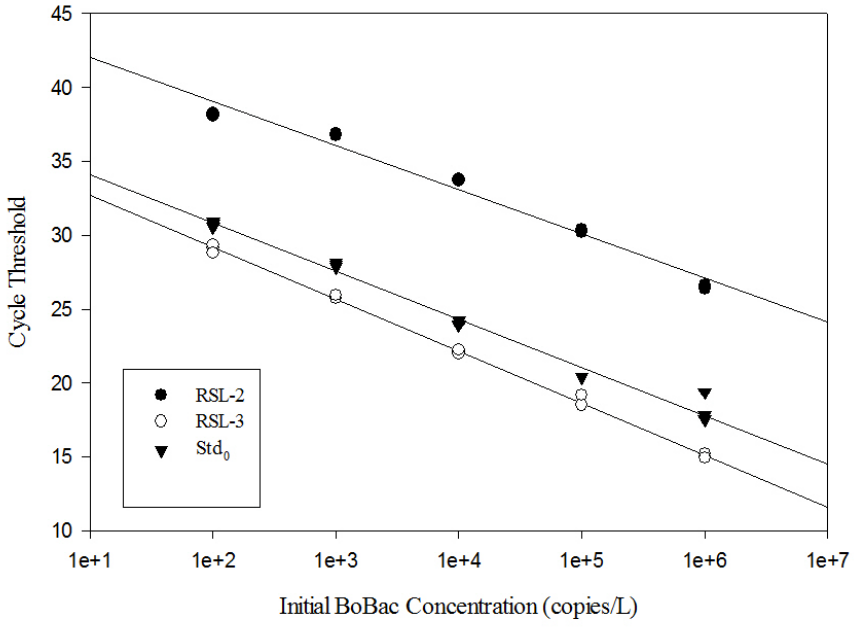

Figure 3.2 Most divergent BoBac standard curves

Fig. 3.3 shows a phylogenetic tree of *Bacteroides* strains found in fecal samples of bovine, human, avian, canine, and equine species. These *Bacteroides* strains were used to develop the AllBac and BoBac molecular markers reported by Layton et al. (2006). Multiple *Bacteroides* strains were sequenced at each sample location. As expected, the number of mismatches occurring within the primer/probe regions increased as the phylogenetic distance increased. For example, sequence RSL-2 had a total of 15 mismatches along BoBac primer and probe regions while RSL-12 had a total of four (see Appendix D). All of the other Riesel sequences had no mismatches along either the AllBac or BoBac primer/probe regions. As was expected, RSL-2 showed the greatest genetic distance within Fig. 3.3, and RSL-12 showed the next most divergence.

It is important to note the various sequences taken from all states varied greatly. See, for example, sequence PA Bo 1-10 at the bottom of Fig. 3.3 and compare it with sequence PA Bo 1-4. Note there are 15 mismatches within the BoBac primer/probe regions of PA Bo 1-10 while no mismatches are within PA Bo 1-4. This is true

throughout the entirety of Fig. 3.3. The 10 sequences taken from Pennsylvania, not to mention all other states, are scattered indiscriminately throughout Fig. 3.3. It is uncertain whether the 10 sequences reported by Layton et al. (2006) were collected from a single locale or at 10 locales across the state. Nevertheless, the results in Fig. 3.3 suggested genetic variation may occur as much within localized samples as between those from broader geographies. Thus, genetic similarity or dissimilarity does not seem to be solely correlated with geographic location at a larger scale. Although geographic variability cannot be ruled out as a potential cause for genetic variability, from the results shown in Fig. 3.3, geographic location is not considered to be the major driving factor behind genetic variability. Although there may be other reasons for creating watershed specific standard curves, geographic variability does not seem to be a sufficient enough problem to merit creating localized standard curves. Seemingly more important is the range of genetic differences within highly localized

Figure 3.3 Phylogenetic dendrogram of *Bacteroides 16S* region. Bovine sequences are labeled with the abbreviated state name corresponding to the location each sample was obtained. The seven Riesel (RSL) standards and the initial standard (Std_0) used by Wagner (2011) are included within the figure and are denoted by the filled circles.

Bacteroides populations (i.e.: within a herd or even individual fecal pats). This begs the question of how to best represent a range of *Bacteroides* subspecies using a single standard curve. Moreover, this reveals the pitfalls of randomly selecting a single *Bacteroides* DNA sequence for use as the gene-copy curve. Figures 3.4 and 3.5 show the importance of creating the standard curve from a sequence most representative of the *Bacteroides* population. However, this is further complicated because enteric bacteria populations are dynamic and may change with time.

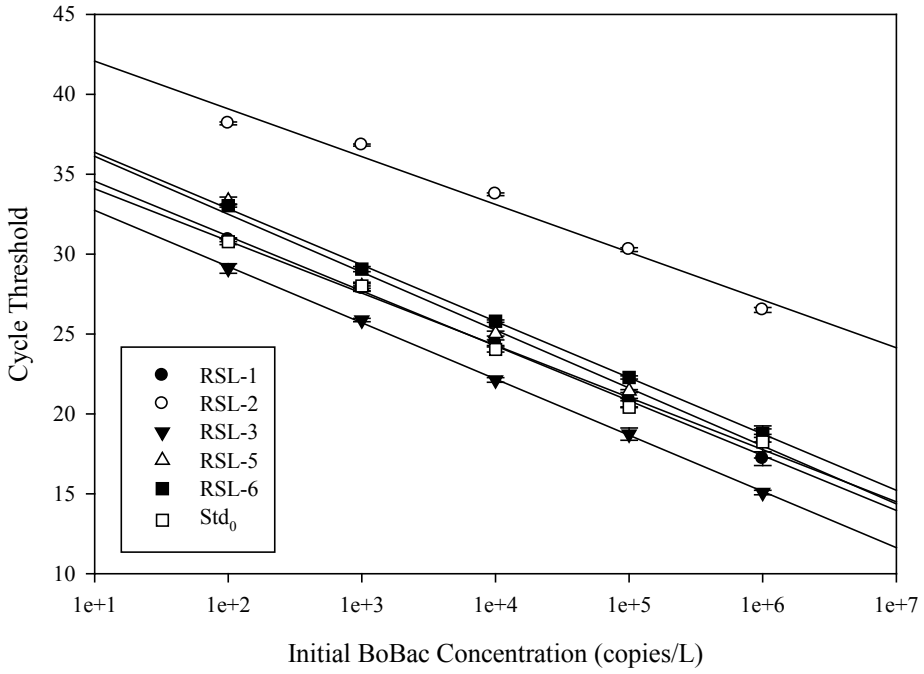

Figure 3.4 All Riesel BoBac standard curves

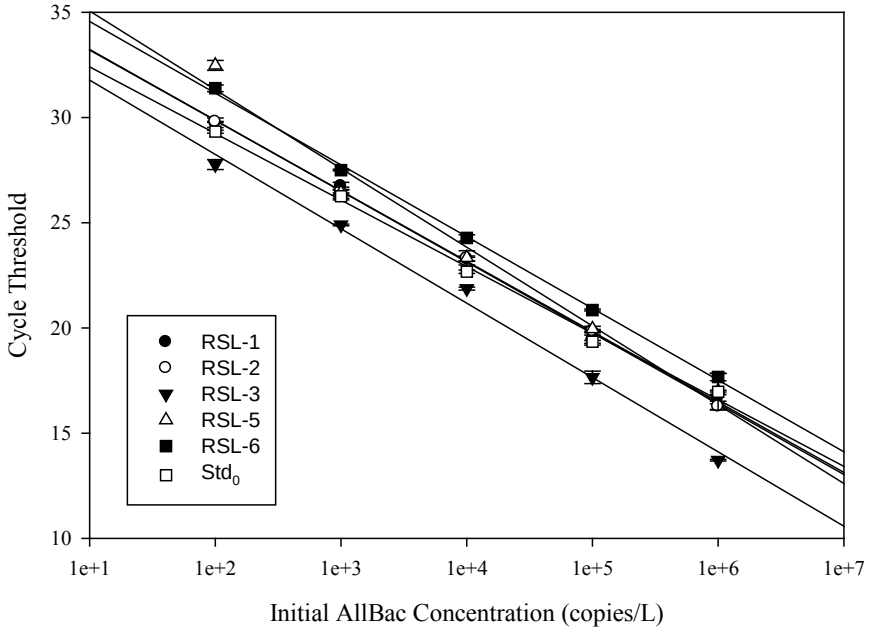

Figure 3.5 All Riesel AllBac standard curves

As concentrations of AllBac/BoBac molecular markers increase within runoff samples, it is reasonable to expect higher *E. coli* concentrations. Wagner 2011 observed a positive correlation between *E. coli* and AllBac/BoBac molecular marker concentrations at the BCSC location; however, little correlation existed at the Riesel and Welder locations. It was supposed using a standard curve developed from the geographical region of the runoff samples may improve the correlation between *E. coli* and AllBac or BoBac markers at that location. However, when new standard curves were developed from the same Riesel cattle herd used in the Wagner (2011) study, no significant correlation improvements between molecular markers and *E. coli* were observed. The R^2 values for all Riesel standard curves remained at 0.2005. Although

the magnitude of the dataset changed when using different standards; ultimately, the regression orientation and R^2 value remained the same. The regression orientation of *E. coli* versus AllBac/BoBac markers could have been expected to change should the slope of the curve change significantly between various standards. It was observed mismatches within the primer/probe regions affect the magnitude of the curve significantly, but the change in slope between various standards were insignificant. Despite the DNA degradation between Std_0 and Wagner (2011), the genetic similarity of Std_0 with most of the other Riesel sequences suggested it fairly represented the *Bacteroides* population at Riesel. From the results shown here, creating a standard curve from fecal samples within the geographical region is not expected to increase correlations between AllBac/BoBac molecular markers and *E. coli* concentrations.

One possible reason for the lack of correlation between *E. coli* and AllBac/BoBac molecular markers observed in the Wagner (2011) study is the *E. coli* concentrations may have varied greatly between BCSC and Riesel locations. *E. coli* concentrations have been shown to vary greatly between the fecal samples of different cows (Omisakin et al., 2003). It is likely *E. coli* do not correlate well with AllBac/BoBac markers because *E. coli* are not necessarily normally distributed between individual cow manure samples. Similar to the *Bacteroides* results above, *E. coli* may show as much variability within locations as between. The results from prescribed grazing study above showed statistically significant differences between *E. coli* concentrations at the BCSC and Riesel locations even when no highly evident differences in treatment occurred. It is not illogical to presume variability between *E.*

coli concentrations are the likely cause for the lack of correlation observed by Wagner (2011). *E. coli* variability is the most likely cause for the lack of correlation between AllBac/BoBac molecular markers and *E. coli* concentrations between the Riesel and Welder locations.

3.5 Conclusions

Base-pair mismatches did occur, and they did effect qPCR efficiencies, thus the null hypothesis was rejected. Within runoff samples using the BoBac assay, fecal pollution load estimations were drastically overestimated by using sequences with more mismatches as the standard curve. Even within the GenBank *Bacteroides* sequences, the AllBac assay showed fewer mismatches on fewer occasions than the BoBac assay. This suggested the AllBac assay may be less problematic than the BoBac assay. As expected, the number of mismatches within the primer/probe regions increased as the phylogenetic distance between *Bacteroides* sequences increased. Genetic diversity, or phylogenetic distance, was observed within samples from all locations. In the same way, genetic similarity was not correlated to a particular geography. This indicated genetic variability within *Bacteroides* populations occurs within a single location and also between locations. From these results, creating standard curves for individual watersheds would not necessarily improve the pollution load estimations, thus the null hypothesis was accepted. When creating a standard curve, seemingly more important is the need to select a *Bacteroides* sequence with no mismatches along the primer/probe regions.

CHAPTER IV

SUMMARY

4.1 Non-Structural BMPs

No significant differences in *E. coli* concentrations were observed between *E. coli* concentrations in runoff from heavily stocked, moderately stocked, or non-grazed pastures when pastures had been destocked for greater than 14 days. While pastures were actively stocked or within 14 days of being destocked, *E. coli* concentrations were significantly higher than destocked pastures. Bacterial concentrations in runoff varied significantly between different stocking rates, but only when sites were stocked when runoff occurred. *E. coli*, *Enterococci*, and fecal coliform concentrations varied greatly between runoff events even when no apparent differences in stocking or timing treatments existed. Background *E. coli* concentrations from non-grazed pastures were also very high and varied greatly between runoff events.

4.2 Structural BMPs

The shade structure reduced cattle's dependence on riparian shade, and both the alternative shade and water BMPs helped improve pasture utilization. The alternative water BMP did not reduce the amount of time cattle spent within the riparian zone for this particular study. In this study, cattle used the riparian zone in this pasture primarily for shade, as the alternative water BMP was not effective at decreasing time cattle spent within the riparian zone despite the secondary water source being well utilized. Results from the riparian rip-rap trials were inconclusive; however, preliminary rip-rap trials

showed larger, 20 to 40 cm (8 to 16 in) diameter, rip-rap was highly effective at modifying cattle trough preference.

4.3 *Bacteroides*

Base-pair mismatches occurred, and significantly effected qPCR efficiencies. Within runoff samples using the BoBac assay, fecal pollution load estimations were drastically overestimated by using sequences with more mismatches as the standard curve. As expected, the number of mismatches within the primer/probe regions increased as the phylogenetic distance between *Bacteroides* sequences increased. Genetic diversity, or phylogenetic distance, was observed within samples from all locations. In the same way, genetic similarity was not correlated to a particular geography. This indicated genetic variability within *Bacteroides* populations occurs within a single location and also between locations. Thus, creating standard curves for individual watersheds would not necessarily improve pollution load estimations.

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APPENDIX A

Grazing management

Location	Site	Start Date	End Date	AU/ha	AUD/ha	ha/AUY
BCSC	BB-1	9/7/10	9/9/10	2.5	4.9	16.4
	BB-2	1/12/09	1/16/09	4.0	16.0	22.8
	BB-2	5/22/09	6/5/09	6.1	79.0	3.8
	BB-2	8/7/09	8/8/09	6.4	6.0	3.6
	BB-2	8/12/09	8/19/09	6.4	46.0	2.5
	BB-2	11/12/09	11/17/09	18.4	90.0	1.5
	BB-2	2/1/10	2/8/10	2.5	17.0	1.5
	BB-2	6/21/10	7/2/10	17.7	194.0	1.7
	BB-2	9/7/10	9/9/10	2.5	4.9	1.2
	BB-2	11/2/10	11/8/10	12.8	86.8	0.9
	BB-2	11/16/10	11/18/10	12.8	22.4	0.9
	BB-2	12/6/10	12/8/10	11.2	22.0	1
	BB-2	12/10/10	12/21/10	21.6	117.7	0.8
	BB-2	6/13/11	6/16/11	24.8	37.3	0.8
	BB-2	10/18/11	10/21/11	7.2	23.1	1.2
	BB-2	10/24/11	10/31/11	8.0	57.6	1
	BB-2	4/11/12	4/15/12	8.8	34.1	2.4
	BB-3	1/12/09	1/16/09	8.0	32.0	11.4
	BB-3	5/22/09	6/5/09	13.4	175.0	1.8
	BB-3	8/7/09	8/8/09	12.8	13.0	1.7
	BB-3	8/12/09	8/19/09	12.8	92.0	1.2
	BB-3	11/12/09	11/17/09	36.8	180.0	0.7
	BB-3	2/1/10	2/8/10	2.5	17.0	0.8
	BB-3	6/21/10	7/2/10	31.7	345.6	1
	BB-3	11/2/10	11/8/10	25.6	173.6	0.5
	BB-3	11/16/10	11/18/10	24.8	43.5	0.6
	BB-3	12/6/10	12/8/10	22.4	43.9	0.6
	BB-3	12/10/10	12/22/10	73.7	267.8	0.4
	BB-3	6/13/11	6/16/11	53.7	82.0	0.4
	BB-3	10/18/11	10/21/11	16.8	54.0	0.7
	BB-3	10/24/11	10/31/11	16.0	115.1	0.6
	BB-3	4/11/12	4/15/12	20.0	77.4	1.1
Welder	WWR-2	6/21/10	6/22/10	2.1	5.1	70.9
	WWR-2	9/11/10	9/30/10	3.4	49.0	6.7
	WWR-2	5/31/11	6/2/11	2.1	11.4	5.6
	WWR-3	12/1/07	2/13/08	0.4	31.0	11.6
	WWR-3	4/18/08	4/28/08	2.6	26.0	6.4
	WWR-3	10/20/08	10/25/08	2.9	15.0	5.1

	WWR-3	4/27/09	5/1/09	3.4	14.0	11.7
	WWR-3	6/21/10	6/22/10	2.6	2.6	140.9
	WWR-3	9/1/10	9/11/10	2.6	25.9	12.8
	WWR-3	5/31/11	6/2/11	1	5.2	70.2
Riesel	W-10	8/10/10	10/29/10	1.2	98.1	3.7
	W-10	12/14/10	4/13/11	1.2	147.1	1.5
	W-10	5/11/11	8/11/11	1.2	112.8	1
	W-10	11/10/11	3/20/12	1.2	160.6	0.9
	SW-17	9/12/07	11/14/07	1.1	70.0	2.6
	SW-17	2/25/08	6/2/08	1.1	109.0	1.7
	SW-17	11/5/08	4/21/09	1.1	185.0	1.6
	SW-17	5/1/09	6/3/09	1.1	37.0	1.7
	SW-17	7/15/09	11/6/09	1.1	126.0	1.1
	SW-17	5/3/10	5/24/10	1.1	23.3	2.3
	SW-17	7/19/10	8/27/10	1.1	43.2	2.5

APPENDIX B

E. coli concentrations with site name and runoff date

Site	Runoff Date	CFU/ 100ml	Flow (L)	Site	Runoff Date	CFU/ 100ml	Flow (L)	Site	Runoff Date	CFU/ 100ml	Flow (L)	Site	Runoff Date	CFU/ 100ml	Flow (L)
BB1	3/25/09	1200	5.9E+03	BB2	5/12/11	13600	2.5E+01	BB3	3/20/12	5300	3.0E+05	SW17	10/13/09	13000	2.2E+05
BB1	4/17/09	1070	3.8E+04	BB2	1/24/12	204	7.8E+02	BB3	3/29/12	105000	3.0E+05	SW17	10/26/09	15000	5.3E+05
BB1	4/18/09	4400	5.0E+04	BB2	1/25/12	13000	6.9E+04	SW12	3/3/08	440	-	SW17	1/16/10	20	-
BB1	4/28/09	7600	1.7E+04	BB2	2/4/12	57000	5.1E+05	SW12	3/6/08	9800	4.7E+04	SW17	1/16/11	200	1.6E+05
BB1	10/4/09	57000	5.7E+03	BB2	2/13/12	30000	-	SW12	3/10/08	2200	1.6E+05	SW17	1/25/12	350	1.6E+05
BB1	10/9/09	36000	1.3E+04	BB2	2/15/12	5900	2.6E+04	SW12	3/10/08	9700	1.4E+05	SW17	2/18/12	330	4.0E+05
BB1	10/13/09	43000	2.6E+05	BB2	2/17/12	640	-	SW12	3/18/08	11200	-	SW17	3/20/12	1900	5.8E+05
BB1	10/26/09	153000	1.9E+05	BB2	2/18/12	3200	1.2E+05	SW12	4/10/08	1470	4.7E+04	W10	1/16/11	6900	1.1E+05
BB1	10/26/09	271000	-	BB2	3/9/12	2100	1.1E+05	SW12	4/10/08	4700	-	W10	1/25/12	720	1.6E+06
BB1	11/21/09	9300	7.6E+03	BB2	3/10/12	4100	1.4E+05	SW12	5/14/08	12900	3.0E+05	W10	2/18/12	17000	3.7E+06
BB1	12/1/09	8100	9.8E+04	BB2	3/20/12	19000	1.6E+05	SW12	5/15/08	4500	1.3E+05	W10	3/10/12	32000	1.9E+05
BB1	1/16/10	410	7.1E+03	BB2	3/29/12	56000	2.0E+05	SW12	3/13/09	260	2.4E+04	W10	3/20/12	47000	4.2E+06
BB1	1/29/10	5400	1.5E+05	BB3	3/13/09	140	5.8E+03	SW12	4/17/09	220	-	WWR1	10/26/09	880	-
BB1	2/4/10	2400	9.0E+03	BB3	3/25/09	6600	4.8E+04	SW12	4/18/09	90	4.7E+04	WWR1	11/20/09	3700	1.8E+05
BB1	5/12/11	9100	-	BB3	3/25/09	7200	-	SW12	4/28/09	110	1.9E+05	WWR1	11/21/09	5500	2.4E+05
BB1	1/24/12	9200	6.9E+01	BB3	3/27/09	2000	1.8E+03	SW12	10/9/09	180	-	WWR1	12/1/09	30000	6.5E+03
BB1	1/25/12	17000	8.6E+02	BB3	4/17/09	450	1.4E+05	SW12	10/9/09	1000	1.5E+05	WWR1	1/15/10	8600	-
BB1	2/4/12	120000	8.4E+03	BB3	4/18/09	2100	1.5E+05	SW12	10/11/09	500	7.8E+04	WWR1	1/16/10	1190	1.5E+05
BB1	2/13/12	2300	1.7E+01	BB3	4/28/09	22000	2.2E+05	SW12	10/13/09	2700	1.3E+05	WWR1	2/5/10	880	1.7E+04
BB1	2/15/12	13200	2.7E+02	BB3	10/4/09	1800	1.2E+05	SW12	10/22/09	2600	2.9E+05	WWR1	2/11/10	5500	3.4E+04
BB1	2/17/12	750	-	BB3	10/4/09	2800	-	SW12	10/26/09	10100	5.6E+05	WWR1	7/1/10	400	5.8E+05
BB1	2/18/12	1360	1.2E+03	BB3	10/9/09	15000	2.0E+05	SW12	10/30/09	5900	1.6E+04	WWR1	9/19/10	330	5.4E+05
BB1	3/9/12	9000	2.5E+03	BB3	10/13/09	5600	5.1E+05	SW12	1/16/10	2800	-	WWR1	9/23/10	2800	9.4E+04
BB1	3/10/12	8000	1.3E+03	BB3	10/26/09	90000	3.8E+05	SW12	1/16/11	1200	4.7E+04	WWR2	10/26/09	710	1.7E+01
BB1	3/20/12	24000	2.6E+03	BB3	10/26/09	45000	-	SW12	1/25/12	1800	2.5E+05	WWR2	11/20/09	350	3.1E+03
BB1	3/29/12	1600	2.4E+03	BB3	11/16/09	800000	7.3E+03	SW12	2/18/12	7500	3.2E+05	WWR2	11/21/09	530	3.5E+03
BB2	3/25/09	1500	1.2E+04	BB3	11/21/09	210000	9.8E+04	SW12	3/10/12	2200	6.4E+04	WWR2	9/19/10	1600	5.1E+04
BB2	3/25/09	1000	-	BB3	11/29/09	87000	9.1E+03	SW12	3/20/12	2800	9.0E+05	WWR2	9/23/10	1700	6.8E+05
BB2	4/17/09	980	1.0E+05	BB3	12/1/09	13300	2.6E+05	SW17	3/3/08	77000	1.5E+03	WWR3	10/26/09	5500	-
BB2	4/18/09	2700	1.2E+05	BB3	1/16/10	830	5.4E+04	SW17	3/6/08	18800	3.2E+04	WWR3	11/20/09	4100	2.5E+05
BB2	4/28/09	12200	1.5E+05	BB3	1/29/10	4300	3.2E+05	SW17	3/10/08	13000	-	WWR3	11/20/09	2400	-
BB2	10/4/09	5400	2.2E+04	BB3	2/4/10	2600	6.7E+04	SW17	3/10/08	17100	4.8E+04	WWR3	11/21/09	7500	3.4E+05
BB2	10/4/09	4400	-	BB3	2/8/10	8100	1.5E+05	SW17	3/18/08	19400	8.0E+04	WWR3	12/1/09	5400	2.3E+04
BB2	10/9/09	21000	8.7E+04	BB3	5/12/11	118000	2.3E+03	SW17	4/10/08	26000	1.4E+04	WWR3	12/17/09	330	8.8E+03
BB2	10/13/09	28000	4.8E+05	BB3	1/24/12	750	1.4E+04	SW17	4/18/08	9900	1.6E+04	WWR3	1/15/10	8500	1.8E+05
BB2	10/26/09	162000	3.5E+05	BB3	1/25/12	8300	8.1E+04	SW17	5/14/08	28000	2.4E+05	WWR3	1/16/10	2700	-
BB2	10/26/09	181000	-	BB3	2/4/12	240000	9.0E+05	SW17	3/13/09	5400	3.7E+04	WWR3	2/5/10	10300	4.2E+04
BB2	11/21/09	58000	6.1E+04	BB3	2/13/12	3900	7.8E+03	SW17	4/17/09	113000	9.6E+04	WWR3	2/11/10	5500	6.8E+04
BB2	12/1/09	10800	2.0E+05	BB3	2/15/12	1700	3.3E+04	SW17	4/18/09	38000	-	WWR3	7/1/10	2600	7.4E+05
BB2	1/16/10	4900	5.3E+04	BB3	2/17/12	1490	-	SW17	4/28/09	29000	1.4E+05	WWR3	9/19/10	390	6.8E+05
BB2	1/29/10	9500	2.8E+05	BB3	2/18/12	270	1.1E+05	SW17	10/9/09	2200	1.1E+05	WWR3	9/23/10	1600	9.1E+04
BB2	2/4/10	8800	5.4E+04	BB3	3/9/12	2200	2.5E+05	SW17	10/9/09	14000	-				
BB2	2/8/10	4400	1.3E+05	BB3	3/10/12	1700	1.2E+05	SW17	10/22/09	32000	2.9E+05				

APPENDIX C

Temperature, relative humidity, and solar radiation data at McGregor Agri-life Research Center

Trial 1

	Temperature		Relative Humidity		
Date	Max	Min	Max	Min	Max Solar Radiation
7-Oct-10	81	42	93	21	896
8-Oct-10	85	45	92	19	890
9-Oct-10	85	48	87	21	879
10-Oct-10	86	49	89	24	873
11-Oct-10	85	57	98	37	778
12-Oct-10	86	63	100	46	535
13-Oct-10	84	58	100	29	843
14-Oct-10	83	56	83	37	801
15-Oct-10	77	46	78	20	859
16-Oct-10	83	40	93	15	859
17-Oct-10	84	46	84	20	850
18-Oct-10	83	54	94	40	704
19-Oct-10	85	63	97	41	785
20-Oct-10	86	60	97	39	808
21-Oct-10	86	59	98	45	599
22-Oct-10	87	58	98	41	764
23-Oct-10	85	67	99	51	403
24-Oct-10	81	62	100	70	519
25-Oct-10	86	63	100	53	509
26-Oct-10	86	65	100	52	671
27-Oct-10	79	59	91	26	781

Trial 2

	Temperature		Relative Humidity		
Date	Max	Min	Max	Min	Max Solar Radiation
26-May-11	99	73	91	13	1162
27-May-11	87	61	72	29	1155
28-May-11	99	66	83	28	1102
29-May-11	98	73	89	33	1088
30-May-11	97	73	89	36	1101
31-May-11	95	75	86	35	907
1-Jun-11	95	74	90	37	1013
2-Jun-11	94	71	89	24	1118
3-Jun-11	97	65	82	29	1107
4-Jun-11	98	68	81	23	1125
5-Jun-11	95	67	93	27	1131
6-Jun-11	99	68	76	28	1030
7-Jun-11	100	68	87	23	1076
8-Jun-11	98	69	80	27	1065
9-Jun-11	96	72	91	33	1103
10-Jun-11	96	72	88	27	1085
11-Jun-11	96	72	91	31	1081
12-Jun-11	97	72	87	31	1104
13-Jun-11	98	72	88	27	1104
14-Jun-11	100	73	84	25	1114
15-Jun-11	101	75	85	23	1129
16-Jun-11	101	76	83	22	1098
17-Jun-11	102	75	85	26	1108
18-Jun-11	103	79	80	23	1082

Trial 3

	Temperature		Relative Humidity		
Date	Max	Min	Max	Min	Max Solar Radiation
18-Nov-11	60	36	62	29	745
19-Nov-11	67	39	69	44	505
20-Nov-11	79	64	87	62	269
21-Nov-11	82	54	89	54	364
22-Nov-11	63	48	97	81	248
23-Nov-11	56	49	97	79	317
24-Nov-11	68	46	94	51	710
25-Nov-11	70	40	96	51	692
26-Nov-11	74	51	95	52	692
27-Nov-11	65	49	97	57	322
28-Nov-11	51	34	65	22	727
29-Nov-11	60	27	77	20	711
30-Nov-11	64	33	81	25	654
1-Dec-11	62	34	85	30	696
2-Dec-11	68	39	93	63	460
3-Dec-11	61	46	98	60	99
4-Dec-11	66	53	95	68	126
5-Dec-11	53	41	95	93	115
6-Dec-11	42	34	95	82	140
7-Dec-11	37	29	87	62	318
8-Dec-11	49	24	84	38	695
9-Dec-11	53	29	87	35	673

Trial 4

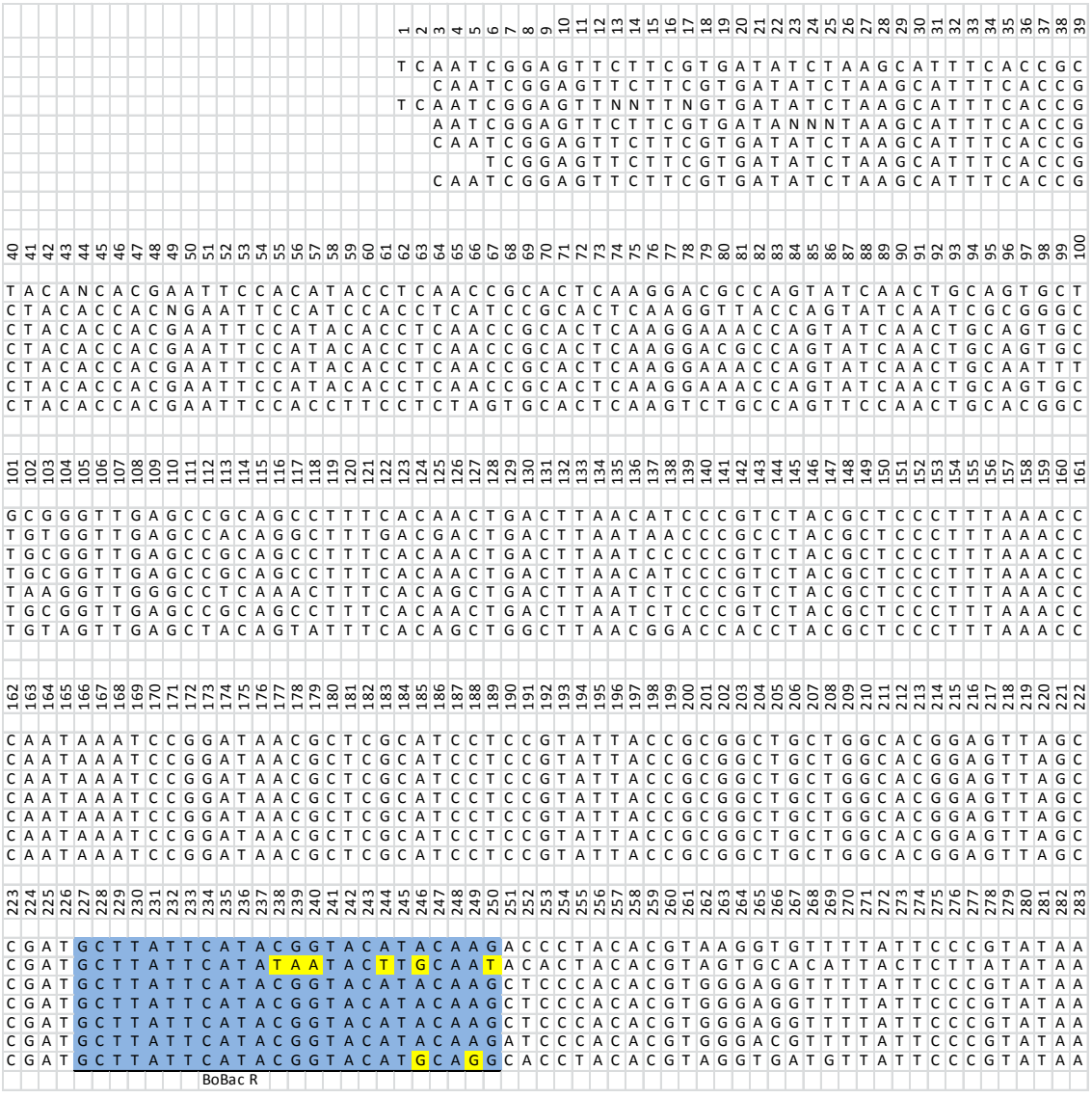
	Temperature		Relative Humidity		
Date	Max	Min	Max	Min	Max Solar Radiation
28-Mar-12	77	58	91	57	965
29-Mar-12	71	61	95	68	260
30-Mar-12	79	63	98	65	926
31-Mar-12	81	65	98	67	913
1-Apr-12	84	63	98	56	1021
2-Apr-12	86	66	96	52	1031
3-Apr-12	82	68	95	51	917
4-Apr-12	80	60	96	71	614
5-Apr-12	76	51	98	58	1003
6-Apr-12	80	57	95	38	1047
7-Apr-12	79	52	96	51	998
8-Apr-12	82	63	97	49	919
9-Apr-12	81	60	96	50	978
10-Apr-12	79	58	98	55	943
11-Apr-12	81	60	95	47	680
12-Apr-12	81	59	96	46	1012
13-Apr-12	80	66	93	53	780
14-Apr-12	81	63	93	59	718
15-Apr-12	81	70	87	64	579
16-Apr-12	74	56	88	42	576
17-Apr-12	72	51	93	53	641
18-Apr-12	79	50	94	29	1108

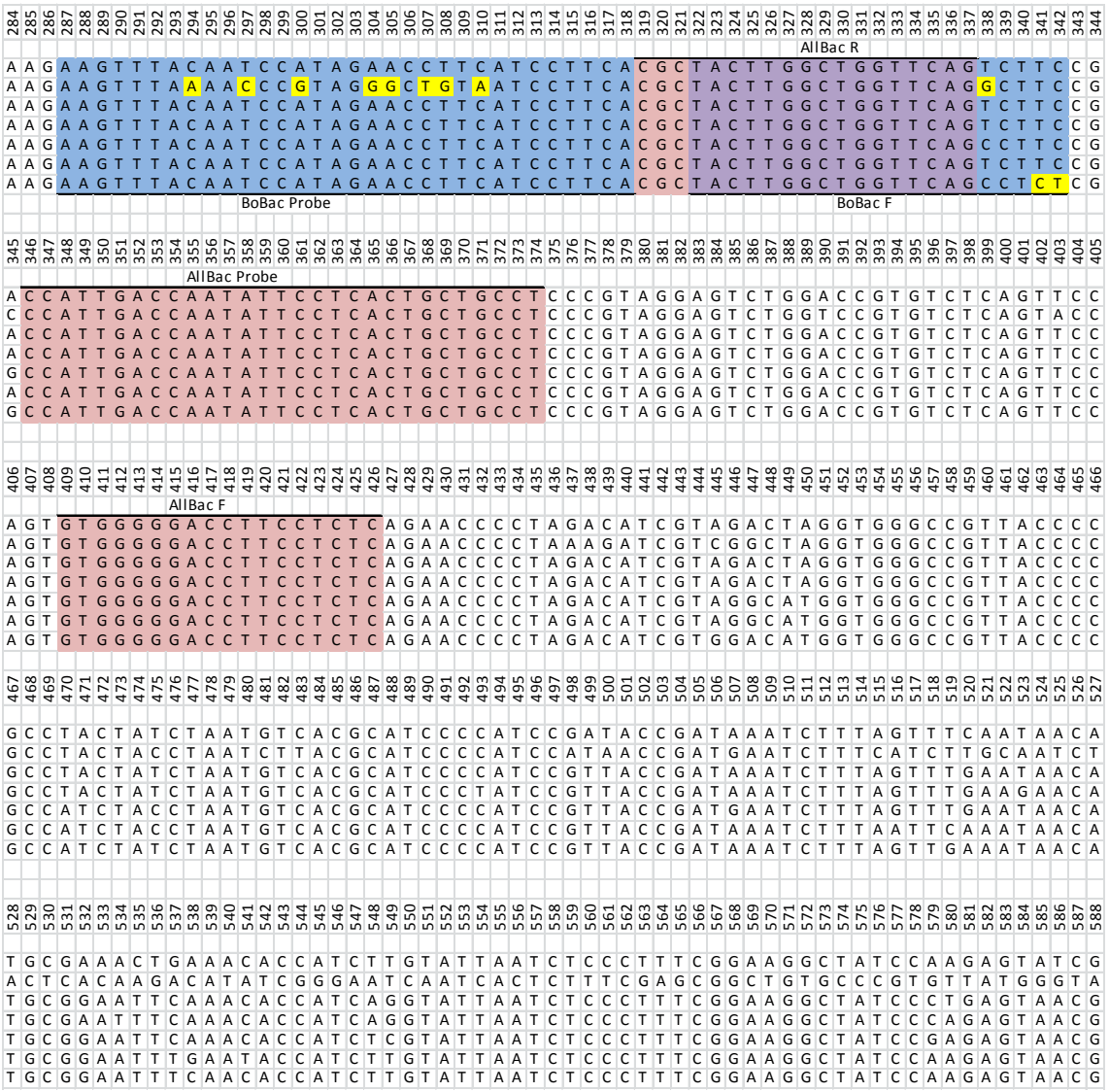
Trial 5

	Temperature		Relative Humidity		
Date	Max	Min	Max	Min	Max Solar Radiation
26-Apr-12	88	63	89	44	1045
27-Apr-12	90	67	90	40	1015
28-Apr-12	87	67	88	37	1051
29-Apr-12	88	68	86	41	926
30-Apr-12	85	69	91	39	890
1-May-12	83	71	92	54	911
2-May-12	86	69	90	45	863
3-May-12	88	70	87	48	812
4-May-12	90	66	95	44	1080
5-May-12	93	71	88	45	1059
6-May-12	92	70	87	40	1035
7-May-12	88	74	74	38	753
8-May-12	91	66	89	33	1076
9-May-12	69	64	94	84	229
10-May-12	80	62	89	41	887
11-May-12	76	59	92	49	539
12-May-12	80	61	97	57	754
13-May-12	79	58	97	46	858
14-May-12	83	59	94	34	1054
15-May-12	81	60	89	39	1113
16-May-12	75	61	95	51	568
17-May-12	86	56	97	31	1129
18-May-12	88	58	94	24	1152

APPENDIX D

Pyro-sequenced *Bacteroides* DNA from Riesel samples

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BoBac Probe
BoBac F
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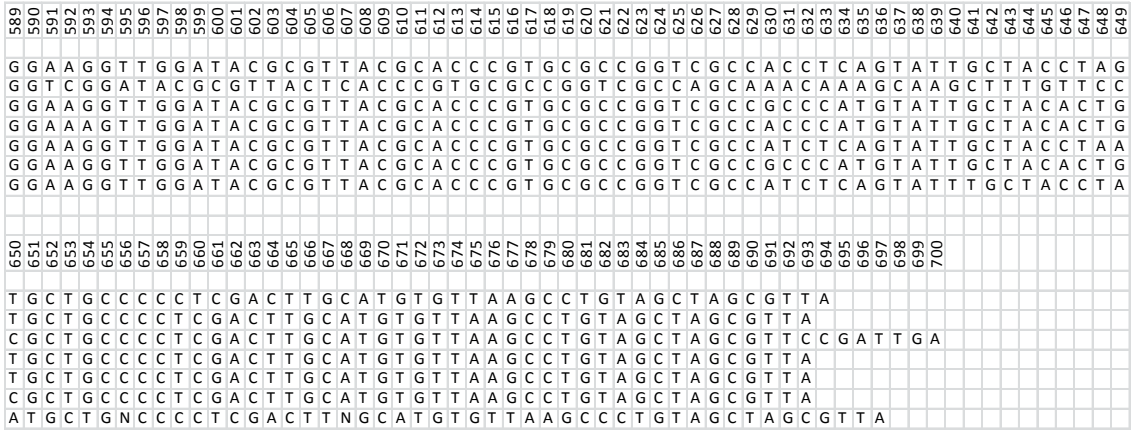
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G G T C G G A T A C G C G T T A C T C A C C C G T G C G C C G G T C G C C A G C A A A C A A A A G C A A G C T T T G T T C C
G G A A G G T T G G A T A C G C G T T A C G C A C C C G T G C G C C G G T C G C C G C C C A T G T A T T G C T A C A C T G
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C G C T G C C C C T C G A C T T G C A T G T G T T A A G C C T G T A G C T A G C G T T C C G A T T G A
T G C T G C C C C T C G A C T T G C A T G T G T T A A G C C T G T A G C T A G C G T T A
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